Improvement of cell-surface adhered cellulase activities in recombinant strains of *Saccharomyces cerevisiae* engineered for consolidated bioprocessing

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I declare that "**Improvement of cell-surface adhered cellulase activities in recombinant strains of *Saccharomyces cerevisiae* engineered for consolidated bioprocessing**" is my own work, that it has not been submitted for any degree or examination in any other university, and that all sources I have used I have used have been indicated and acknowledge by complete references.

Signature:

Date: April 2021

Table of Contents

a. Acknowledgements i

b. List of Abbreviations ii

c. List of Figures iv

d. List of Tables v

e. Abstract vi

1. Chapter 1: Literature Review 1

1.1. Introduction 1

1.2. Bioenergy 4

1.2.1. First Generation Biofuels 6

1.2.2. Second Generation Biofuels 7

1.2.3. Bioethanol 8

1.3. Lignocellulosic Biomass 9

1.3.1. Hydrolysis of Lignocellulosic Biomass to Ethanol 11

1.4. Enzymes Required for Cellulose Hydrolysis 13

1.4.1. Endoglucanase (EG) 14

1.4.2. Cellobiohydrolase (CBH) 15

1.4.3. β-glucosidase (BGL) 16

1.5. Saccharification and Fermentation Bioprocesses 17

1.5.1. Separate (Sequential) Hydrolysis and Fermentation (SHF) 17

1.5.2. Simultaneous Saccharification and Fermentation (SSF) 18

1.5.3. Simultaneous Saccharification and Co-fermentation (SSCF) 19

1.6. Consolidated Bioprocessing (CBP) 19

1.7. Recombinant Cellulolytic Strategy: Engineering Yeast Strains 22

1.7.1. Secreted Cellulases 23

1.7.2. Cell-Tethered Cellulases 23

1.8. *Saccharomyces cerevisiae* – Industrial and Laboratory Workhorse 28

1.9. Potential of *S. cerevisiae* as a CBP organism 28

1.10. Problems Associated with Heterologous Cellulase Production 29

1.11. Strain Engineering for Enhanced Expression of Cellulases 31

1.11.1. Protein Secretory Pathway 32

1.11.2. Protein Secretion Stressors 35

1.11.3. Potential Gene Targets for Improved Heterologous Secretion 37

1.11.4. SNARE proteins 38
1.12. Objectives of the Study 42
2. Chapter 2: Materials and Methodology 44
2.1. Media and Culturing Conditions 44
2.2. Heterologous Strain Construction 44
2.3. PCR Confirmation 47
2.4. Enzyme Assays 48
2.5. Cellulose Conversion 49
2.6. Growth Analysis 49
2.7. Evaluation of Fermentation Stressors 50
2.8. Fermentation of Crystalline Cellulose 50
2.9. High-performance liquid chromatography (HPLC) 51
3. Chapter 3: Results and Discussion 52
3.1. Enzyme Activity 52
3.1.1. Strain Expressing BGL Only (Parental Strain: BY-BG-SS) 52
3.1.2. Strain expressing BGL, EG and CBH (Parental Strain: EG-d-CBHI-d-CBHII-d) 54
3.1.3. Ratio optimized strain expressing BGL, EG and CBH (Parental Strain: δ-integrated A26) 56
3.2. Cellulose Conversion 58
3.3. Growth Analysis 60
3.4. Fermentation Derived Stress Tolerance Testing 63
3.4.1. Heat Tolerance 64
3.4.2. End Product Inhibition 66
3.4.3. ER Stress Tolerance 67
3.4.4. Osmotic Tolerance 68
3.5. Fermentation of Crystalline Cellulose 69
3.6. Gene Combination Studies 71
4. Chapter 4: Summary and Conclusion 75
4.1. Future Prospects 78
5. References 79
6. Appendix 89

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b. List of Abbreviations

1G First generation

2G Second generation

ATP Adenosine Triphosphate

BGL Beta (β)-Glucosidase

BiP Binding Immunoglobin Protein

CBH Cellobiohydrolase

CBP Consolidated Bioprocessing

CCS Carbon capture and sequestration

COPII Coat Protein Complex II

EG Endoglucanase

ER Endoplasmic Reticulum

ERAD Endoplasmic Reticulum Associated Degradation

ERO1 Endoplasmic Reticulum Oxidoreductase 1

FCEV Fuel Cell Electric Vehicles

GHG Greenhouse gas

GPI Glycosylphosphatidylinositol

GPI-CWP GPI-anchored cell wall protein

IPCC Intergovernmental Panel on Climate Change

IRE1 Inositol-requiring Enzyme 1

LCB Lignocellulosic biomass

mRNA messenger ribonucleic acid

MU Methylumbelliferone

MULac 4-Methylumbelliferyl β-D-lactopyranoside

NREL National Renewable Energy Laboratory

NSF N-ethylmaleimide-sensitive factor

PASC Phosphoric Acid Swollen Cellulose

PDI Protein Disulfide Isomerase

PV Photovoltaic

QC Quality Control

RES Renewable Energy Source

RFP Red Fluorescence Protein

RI Refractive Index

ROS Reactive Oxygen Species

SCF Separate (or sequential) Hydrolysis and Fermentation

SNARE Soluble N-ethylmaleimide-sensitive factor attachment receptor proteins

SSF Simultaneous Saccharification and Fermentation

SSFC Simultaneous Saccharification and Co-fermentation

UNFCCC United Nations Framework Convention on Climate Change

UPR Unfolded Protein Response

YP Yeast Peptone

YPD Yeast Peptone Dextrose

c. List of Figures

Figure 1.1: A schematic diagram of the typical biorefinery process steps currently in place (Zhu & Zhuang, 2012)……..……..……..5
Figure 1.2: Diagrammatic illustration of the structure of lignocellulose and its biopolymers (Hernandez-Beltrán, et al., 2019)…… ……..……..10
Figure 1.3: Diagrammatic representation of the enzymatic hydrolysis of cellulose by the synergistic action of cellulases (Ratanakhanokchai, et al., 2013)……..……..……...13
Figure 1.4: Common process configurations for the conversion of lignocellulosic biomass to bioethanol (Zhang & Lynd, 2008)……..……..17
Figure 1.5: The two major strategies for engineering cellulolytic *Saccharomyces cerevisiae* for cellulosic ethanol production are via either cell-surface display or secretion of enzymes (Liu, et al., 2016) ……23
Figure 1.6: Schematic representations of cellulase expressing plasmids. (A) Plasmid for single copy integration. (B) Plasmid for δ-integration (Yamada, et al., 2010)……..……...27
Figure 1.7: Schematic overview of the secretory pathways in yeast (Sheng, et al., 2017)…….....……...32
Figure 1.8: Schematic representation of protein folding, quality control, degradation and secretion in yeast (Gasser, et al., 2008)…… ……..……34
Figure 1.9: A diagram representing typical bottlenecks in the secretory pathway of heterologous proteins in yeast. The main membrane trafficking and vacuolar protein sorting pathways are indicated (Idris, et al., 2010)36
Figure 1.10: Illustration of various engineering attempts that have been made on the yeast secretory pathway; with green boxes listing gene overexpression and red boxes genes that were deleted (Kroukamp, et al., 2018)...39
Figure 3.1: The β-glucosidase (p-nitrophenyl-β-D-glucopyranoside) activity profiles of the recombinant Saccharomyces cerevisiae BYBGSS strains transformed with one of seven genes reported to improve cellulase secretion. Values obtained were normalised using dry cell weight (DCW) of each strain and the parental BYBGSS strain was used as reference. Reference activity indicated by red dotted line. All values represent mean values of assays conducted in triplicate with error bars indicating the standard deviation from the mean value for each strain……53
Figure 3.2: Enzyme activity profiles of the recombinant Saccharomyces cerevisiae ECC and ΔA26 strains transformed with one of the seven genes reported to improve cellulase secretion after 72 hours of cultivation. (A&D) β-glucosidase activity on p-nitrophenyl-β-D-glucopyranoside. (B&E) endoglucase activity on carboxymethyl cellulose. (C&F) cellobiohydrolase activity on 4-methylumbelliferyl-β-D-lactoside. Values obtained were normalised using dry cell weight of each strain and the parental strains used as reference (activity indicated by red line). All values represent mean values of assays conducted in triplicate with error bars indicating the standard deviation from the mean value for each strain……55
Figure 3.3: The hydrolysis of crystalline avicel by the recombinant Saccharomyces cerevisiae strains after 24 and 48 hours of incubation. (A) ECC transformants (B) ΔA26 transformants. Values obtained were normalised using dry cell weight (DCW) of each strain and the parental ECC and ΔA26 strain were used as reference (comparison of activity to all other strains shown using red dotted line). All values represent mean values of assays conducted in triplicate with error bars indicating the standard deviation from the mean value for each strain……..……58
Figure 3.4: (A) Growth kinetics of BYBGSS parental strain and recombinant BYBGSS strains transformed with PSE1, SEC9 or SED5 SNARE genes. (B) Growth kinetics of ECC parental strain and recombinant ECC strains transformed with PSE1, SEC9 or SED5 SNARE genes. (C) Growth kinetics of ΔA26 parental strain and recombinant ΔA26 strains transformed with SNC1, SSO1 or SED5 SNARE genes. Stains used had been identified to have superior enzyme activities over all other transformants. Strains were cultivated over a period of 39 hours in yeast peptone dextrose media and absorbance recorded at 600nm (OD600). All values represent mean values of cultures grown in triplicate with error bars indicating the standard deviation from the mean value for each strain…..62
Figure 3.5: Stress plate assays to test for fermentation associated stresses after 48 hours of cultivation for BYBGSS, ECC strains and ΔA26 transformant strains. All cultures were standardized to an OD600nm of 1.4 after which 3µL of tenfold serial dilutions of the cultures were spotted on yeast peptone dextrose agar supplemented with various stressors as indicated. ……..…..65
Figure 3.6: The enzyme activity profiles of the previously identified ECC strains transformed with SEC9 and PSE1 and ΔA26 strains containing SSO1 and SNC1 further transformed to overexpress the SED5 SNARE protein. (A&D) β-glucosidase activity on p-nitrophenyl-β-D-glucopyranoside. (B&E) endoglucanase activity on carboxymethyl cellulose. (C&F) cellobiohydrolase activity on 4-methylumbelliferyl-β-D-lactoside. Values obtained were normalised using dry cell weight (DCW) of each strain and the parental ECC and ΔA26 strain was used as reference. All values represent mean values of assays conducted triplicate with error bars indicating the standard deviation from the mean value for each strain……..…72

Figure A1: Calibration curve of 4-Nitrophenyl used to determine unknown concentrations of released 4-NP from which β-glucosidase activity was determined……..……89
Figure A2: Calibration curve of 4-Methylumbelliferyl used to determine unknown concentrations of released methylumbelliferyl from which cellobiohydrolase activity was determined. Measurements taken using (A) clear bottom 96-well microtitre plates and (B) black bottom 96-well microtitre plates..…89
Figure A3: Calibration curve of glucose used to determine unknown concentrations of reducing sugars from which endoglucanase activity was determined……..90
Figure A4: 1% (w/v) agarose gel electrophoresis of BYBGSS PCR amplified products using phosphoglycerate kinase 1-specific PCR primer sets to confirm the presence of relevant SNARE genes transformed into each BYBGSS strain. Each lane contains the native phosphoglycerate kinase 1 gene at ~1300bp and the additional phosphoglycerate kinase 1 gene. Lane 1 shows the 1kb DNA marker, Lanes 2-8 are the BYBGSS transformants, Lane 9 is the negative control, and Lane 10 BYBGSS parental strain used as the control strain. Asterisk (*) indicates genes that had not been successfully amplified……90
Figure A5: 1% agarose gel electrophoresis of ECC PCR amplified products using phosphoglycerate kinase 1-specific PCR primer sets to confirm the presence of relevant SNARE gene transformed into each ECC strain. Each lane contains the native phosphoglycerate kinase 1 gene at ~1300bp and the additional phosphoglycerate kinase 1 gene. Lane 1 shows the 1kb DNA marker, Lanes 2-8 are the ECC transformants, Lane 9 is the negative control, and Lane 10 ECC parental strain used as the control strain. Asterisk (*) indicates genes that had not been successfully amplified……...91
Figure A6: 1% agarose gel electrophoresis of ΔA26 PCR amplified products using phosphoglycerate kinase 1-specific PCR primer sets to confirm the presence of relevant SNARE gene transformed into each ΔA26 strain. Each lane contains the native phosphoglycerate kinase 1 gene at ~1300bp and the additional phosphoglycerate kinase 1 gene. Lane 1 shows the 1kb DNA marker, Lanes 2-8 are the ΔA26 transformants, Lane 9 is the negative control, and Lane 10 ΔA26 parental strain used as the control strain. Asterisk (*) indicates gene that had not been successfully amplified……...91

d. List of Tables

Table 1.1: Advantages and disadvantages of S. cerevisiae as a potential CBP organism……..30
Table 1.2: SNARE proteins shown to have a positive effect on the secretion of cellulases when overexpressed in recombinant cellulolytic yeast strains……..40
Table 2.1: Description of yeast strains used in this study……....45
Table 2.2: Description of plasmids used in this study……..…47
Table 2.3: Primers used for PCR amplification of transformants generated in this study……47
Table 3.1: Enzyme activity across all strains following a 72 hour incubation and post 48 hours of cellulose conversion as compared to the parental strains. Percentages are an indication of improvements or reductions in activity after expression of the gene indicated, when compared to the parental strains. Green cells represent an increase that had exceeded 10%, while red is an indication of a reduction in activity exceeding 10%.....……...60
Table 3.2: Composition of 96-h fermentation broth samples detected by high-performance liquid chromatography. The parental ECC and ΔA26 strain were included to be used as reference. All values represent mean values in g/L of the fermentations conducted in triplicate after 96 hours. Numbers in brackets represents the standard deviation from the mean value of each sample……70

e. Abstract

Consolidated bioprocessing (CBP), in which a single organism in a single reactor is responsible for the conversion of pretreated lignocellulosic biomass to bioethanol, remains an attractive option for production of commodity products if an organism fit for this process can be engineered. The yeast *Saccharomyces cerevisiae* requires engineered cellulolytic activity to enable its use in CBP production of second generation bioethanol. Current recombinant yeast strains engineered for this purpose must overcome the drawback of generally low secretion titres. A promising strategy for directly converting lignocellulose to ethanol is by displaying heterologous cellulolytic enzymes on the cell surface by means of the glycosylphosphatidylinositol (GPI) or similar anchoring systems. Recently, a strain producing cell-adhered enzymes in a ratio-optimized manner was created that showed significant crystalline cellulose hydrolysis. However, cellulase-displaying yeast strains still secrete levels of enzyme that are insufficient for complete cellulose hydrolysis. Soluble *N*-ethylmaleimide-sensitive factor attachment receptor proteins (SNAREs) are crucial components of trafficking yeast proteins and are required at most membrane fusion events in the cell. SNAREs facilitate fusion between the protein transport vesicles, numerous membrane-enclosed organelles and the plasma membrane. Previous studies have found that heterologous protein secretion levels were increased when overexpressing certain SNARE proteins. This study aimed to improve the amount of cell-adhered cellulase activities of recombinant *S. cerevisiae* strains through over-expression of SNARE encoding genes identified in previous studies. The results showed significant increases in cellulolytic activity for all three cell-adhered cellulase enzyme types. Cell-adhered cellobiohydrolase activity could be improved by up to 101%, beta-glucosidase activity by up to 99% and endoglucanase activity by up to 231%. Improved hydrolysis of crystalline cellulose of up to 424% was also observed, without causing any additional stress to the cells. Moreover, an improvement in resistance to fermentation derived stressors was also noted in some strains, showing characteristics considered to be attractive for use in second generation bioethanol production. Upon identifying genes that had shown to have a positive effect on enzyme activity, over expressing and combining these genes resulted in further increased enzyme activity for some strains. These strains could signify a step towards a more efficient cellulose hydrolysing organism for use in second generation biofuel production.

1. Chapter 1: Literature Review

1.1. Introduction

Growing global population heavily relies on fossil fuels as a major energy source and the depletion of fossil fuels and negative environmental impacts associated with their use has led to the development of alternative energy sources (Sakwa, *et al*., 2018). More specifically, the decline of oil reserves, political instability in the major oil-producing parts of the world, and climate changes due to the excess carbon dioxide (CO_2) released from fossil fuels has led to renewed interest in the conversion of renewable biomass as a source of liquid fuels and other oil-derived products (van Zyl, *et al*., 2012). Biofuels are produced from naturally occurring biomass and can be used as substitutes for fossil fuels (Hasunuma & Kondo, 2012). The substantial attention given to the utilization of biomass as the starting material for various chemicals, as well as for the production of biofuels, is mainly due to the many advantages it has over fossil fuels. The utilization of biomass, especially cellulosic materials, has gained attention due to it being abundantly available, inexpensive, renewable, and having favourable environmental properties (Yamada, *et al*., 2010). Additionally, the combustion of biofuels derived from cellulosic biomass leads to lower sulphuric and aromatic exhaust emissions compared to typical fuels, while providing similar properties in terms of fuel efficacy. This ultimately leads to various governmental programs promoting biofuels (Saini, *et al*., 2014; Lamb & Austbø, 2020).

The increasing role that biofuels have played towards the global energy supply over the past three decades can be attributed to, among other things: (i) energy security, (ii) improved trade balances caused by limiting oil imports, (iii) socio-economic development of developing nations, (iv) concern over fossil fuel reserves, and (v) the need to mitigate greenhouse gas (GHG) emissions (den Haan, 2018). Vast reductions in GHG emissions can be accomplished by either decarbonizing fuels or vehicle technologies, or a combination of both. Fuels based on renewable energy sources (RES) such as biofuels, electricity, and hydrogen generation from RES are all possible avenues to deal with the current transport environmental problems, with different vehicles such as electric vehicles (EV) and fuel cell electric vehicles (FCEV) having the potential to use lower- or zero-carbon energy sources (Li, *et al*., 2018).

The Intergovernmental Panel on Climate Change (IPCC) indicated that for 2020, 2030 and 2050 biofuel use should increase to 2%, 5.1%, and 26.3% respectively to follow the appropriate path in curbing emissions (Fortuna, 2018). Electricity's involvement in the transport mix should also increase to 1.2%, 5%, and 33% respectively for the same periods, meaning that by 2030 biofuel-powered vehicles would continue to be as significant as e-cars. Electromobility and biofuels have been singled out by the IPCC as the two major initiatives of the transport sector decarbonisation and it is expected that biofuels will displace more oil in 2020 and 2030 than renewable electricity will, having the two solutions roughly equal in scale by 2050. Even though electromobility has significant interest in recent years, most of the transport fleet is still expected to be powered by fossil fuels. It is important to note that EVs are not zero-emissions vehicles as they just shift the pollution away from the car to where the power is being produced, with most being powered by burning coal to create the electricity needed to power these vehicles, which is dirtier and more environmentally destructive than burning gasoline unless powered by off-grid sources (Fischer & Keating, 2017). Even if there were to be a massive replacement of existing light vehicles by EVs, it is believed that the cargo and aviation sectors will be using biofuels for a long time to come given that electricity is not a viable option in aviation due to battery sizes and capacities (Pekkala, 2016; Rocha, 2017). Ethanol remains the most prominent biofuel, and is expected to play a bigger role when compared to renewable energy powered electromobility in the next 10 years (Fortuna, 2018). Since replacing two thirds of the cars with EVs is not an option, the demand for biofuels is clear (Pekkala, 2016). The development of a low-GHG gasoline replacement biofuel would allow substantial emission reductions and petroleum savings without the need for new vehicle technologies or expensive refuelling infrastructure (Reichmuth, *et al*., 2013). This is due to biofuels' general compatibility with existing liquid transport fuel and compatibility with existing transportation infrastructure, setting biofuels apart from other alternative energy sources (Saini, *et al*., 2014; Li, *et al*., 2018).

Current biofuel production is almost entirely first generation (1G) and derived from corn and sugarcane to produce ethanol or vegetable oils to produce biodiesel (Chandel, *et al*., 2019). While there has been strong support shown from agricultural interest groups, due to 1G biofuels availability constraints, sustainability concerns, public opposition, and basic economics, greater interest has been shifted towards second generation (2G) biofuels derived from non-edible lignocellulosic residues and wastes. This is mainly because of their potential to offer significant

volumes of low-GHG hydrocarbon fuels at scale, which aids in avoiding many concerns associated with 1G biofuels (Hannula & Reiner, 2019). Comprehensive sustainability requirements are crucial to ensure 2G biofuels can provide substantial GHG emissions reductions without negatively impacting food security, biodiversity, or society (Webb & Coates, 2012). It is for this reason that thorough research of transport biofuel development trends is needed, aimed at identifying possible improvement plans for efficiency and further cost reductions along the production chain for different biofuels. This will ensure a stable and attractive investment environment for biofuel commercialization (Li, *et al*., 2018).

The production of biofuels from cellulosic materials is also able to make important contributions to rural economic development and enhanced sustainability of agricultural landscapes in both developed and developing economies (Lynd, *et al*., 2017). In terms of total energy production, bioethanol closely compares with current fossil fuel sources, with the added advantage of a reduced environmental impact. The problem arises when taking into account the high cost of exogenous enzymes required for biomass hydrolysis which represents one of the most important techno-economical hurdles for the large-scale production of bioethanol (Sakwa, *et al*., 2018). This review will expand on current technologies for the production of bioethanol from lignocellulosic biomass and methods by which this can be enhanced, with specific focus on improved yeast strains used in the fermentative production of bioethanol.

1.2. Bioenergy

During the 20th century, fossil-based sources became the primary supply of energy for transportation, continuing into the 21st century, with most transport vehicles running on fossil fuel derivatives (Lamb & Austbø, 2020). The magnitude at which these energy sources are being used to fulfil the global energy demand has led to an increase in the concentrations of CO_2 in the atmosphere, consequently provoking climate change. This rise in CO_2 concentration has advanced the need to overcome one of the greatest challenges of the 21st century, which is to meet the growing demand for energy for transportation, heating, and industrial processes, as well as to provide raw materials for chemical industries in sustainable and environmentally friendly ways (Saini, *et al*., 2014).

At the 2015 United Nations Framework Convention on Climate Change (UNFCCC) conference in Paris, it was agreed upon by attending parties to keep climate warming well below 2°C relative to pre-industrial levels as well as to attempt to further limit the increase in temperature to 1.5°C. Scientific evidence strongly supports the need for this dual temperature limit by showing that the legally binding 2°C boundary already corresponds to greatly increased risks of catastrophic tipping points with a negative impact on society, economy, and environment (van Meerbeek, *et al*., 2019). The IPCC warns that GHG emissions will continue to increase drastically if additional, far-reaching policies and mitigation measures are not put into place. Governmental actions and incentives have also been put in place to establish greater energy independence, subsequently promoting research on environmentally benign and sustainable alternatives (Hasunuma & Kondo, 2012).

Biofuels have emerged as an ideal alternative to fossil fuels, playing a significant role in many scenarios to limit climate change and meet the requirements set out during the Paris conference (Saini, *et al*., 2014). It is the most established and arguably most promising option available for decarbonizing aviation, ocean shipping, and a substantial fraction of long-haul trucking, without which the 2˚C target agreed to is likely not attainable (Lynd, *et al*., 2017). It contributes to poverty reduction in developing countries; meets energy requirements, cost-effectively delivering energy in a variety of forms that society demands, for example, liquid and gaseous fuels for heat and electricity; and it also has the ability to develop otherwise unproductive and degraded lands, increasing diversity, soil fertility, and water retention (Lamb & Austbø, 2020; Azhar, et al., 2017).

Bioenergy accounted for half of all renewable energy consumed in 2017, providing four times the contribution of solar photovoltaic (PV) and wind combined (International Energy Agency, 2018). Most bioenergy delivers heat in buildings and industry, but it is also expected to account for 3% of electricity production and around 4% of transport energy demand in 2023 (Reid, *et al*., 2020). The concept of producing cellulosic biofuel, bioproducts, and chemicals through a biorefinery using lignocelluloses has been around for 70 years or more. The recent interest in this old concept arises from the promises of mitigating climate change by substituting some petroleum or other fossil fuel energy with biomass energy, in addition to advances in biotechnology in the past three decades (Zhu & Zhuang, 2012).

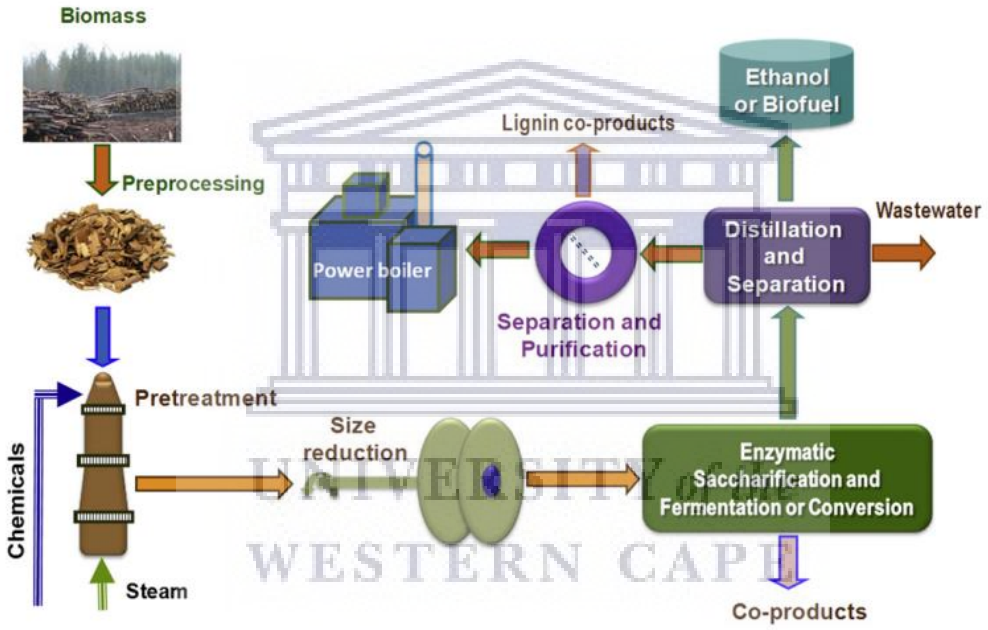

Figure 1.1: A schematic diagram of the typical biorefinery process steps currently in place *(Zhu & Zhuang, 2012).*

However, the realization of the ideal biorefinery concept remains a challenge with economic performance being a primary barrier to commercial deployment (Zhu & Zhuang, 2012). Above is a schematic representation of the typical biorefinery setup currently in place (Figure 1.1). Biofuels produced in these biorefineries are expected to become some of the major sources of renewable energy and mainly include cellulosic ethanol, biodiesel, and biogas (Smekenov, *et al*., 2020). Several studies have suggested that biofuel from lignocellulosic biomass (LCB) can be sustainably produced using modern technology coupled with sound policies (Zhu & Zhuang, 2012). The price

of petroleum is dictated by many factors other than the actual production and delivery costs, such as investor speculation, geopolitics, and government tax policies and subsidies in different forms. Therefore, comprehensive government energy policies and funding strategies are vital for the successful development of a vibrant bio-based economy in addition to advancing biorefinery technologies (Zhu & Zhuang, 2012). Bioenergy will also remain the largest source of renewable energy because of its widespread use in heat and transport. These are sectors in which other renewable energy sources do not currently play a significant role. Wastes and residues will offer low lifecycle GHG emissions and mitigate concerns over land-use change. This represents enormous potential for the entire bioenergy sector, while simultaneously improving waste management and air quality (Fortuna, 2018).

1.2.1. First Generation Biofuels

Conventional biofuel technologies or 1G biofuels are most commonly produced from sugar and starch-rich feedstock, such as cane juice (in Brazil) and molasses (in India) or corn (in the US) (Li, *et al*., 2018). Bioethanol, conventional biodiesel, and biogas all have well-established commercial scale production processes. 1G bioethanol still represents the bulk of ethanol produced globally with the US as the world's leader, producing about 60 billion litres alone with more than 40% of the US corn crop being used to produce bioethanol. When combined with the 26 billion litres produced from sugar cane by Brazil, the two countries produce about 85% of the world's bioethanol (Walker & Walker, 2018). Even whilst being produced at such a large volume, bioethanol production from 1G technologies will likely increase, exceeding 100 billion litres by 2022 (Saini, *et al*., 2014).

Taking into account the increasing global population and concomitant increase in demand for food and energy, 1G bioethanol is not a practical solution to fulfil the goal of sustainable transportation fuels aiming to alleviate the world's dependence on crude oil. Furthermore, the debate regarding the production of 1G bioethanol and its impact on food and water security have persisted throughout the past decade (Xia, *et al*., 2019). Current processes are mainly based on easily fermentable feedstocks that cannot be scaled up to the enormous volumes required without seriously interfering with the human food supply (Valenzuela-Ortega & French, 2019). Additionally, there have been reports regarding the negative impact on biodiversity, and these impacts may even lead to deforestation to gain more farmland (Saini, *et al*., 2014).

It should also be noted that the feedstock used during the production of 1G biofuels contributes 70% of the final cost of the bioethanol (Favaro, *et al*., 2019). Therefore, it is crucial to have an efficient conversion of the raw material into 1G bioethanol for the process economy. Additionally, the production costs from sugar and starch are sensitive to feedstock prices, which have been very volatile in recent years. Biodiesel is produced from raw vegetable oils derived from soybean, canola, palm oil, or sunflowers, as well as from animal fats and used cooking oil. These oils and fats are then converted to biodiesel using methanol or ethanol. Thus, the profitability of conventional biodiesel production is also sensitive to feedstock prices and availability (Li, *et al*., 2018). It is therefore crucial to investigate other potential feedstocks that would be more feasible and less taxing on food security.

1.2.2. Second Generation Biofuels

An alternative option is the use of renewable non-edible plant-derived materials to produce biofuels (Valenzuela-Ortega & French, 2019). Biofuels derived from these sources are referred to as advanced biofuels or are more commonly known as 2G biofuels (Li, *et al*., 2018). Renewable plant biomass refers to cheap and abundant non-food lignocellulose-rich materials available from the plants. 2G biofuels include hydrogen, bio-oils, biogas, alcohols, as well as, biodiesel. There are two fundamentally different approaches to produce these 2G biofuels, namely biological and thermochemical processing though the biologically mediated processes are generally considered more economically viable (Saini, *et al*., 2014; Ojeda, *et al*., 2011).

Commercial 2G bioethanol facilities are slowly becoming operative and attempting to deliver on the promise of cellulosic bioethanol (Lynd *et al.,* 2017). Motivated by the 1970s energy crisis, extensive effort has been devoted to cellulosic ethanol, along with pilot and demonstration plants being established to test its techno-economic viability. Unfortunately, 2G fuel ethanol is not yet economically competitive for large-scale commercial production (Xia, *et al*., 2019). 2G biofuels have been developed rapidly over the past few years, however, to be economically feasible, their yield, titre, and productivity require further improvements (Li, *et al*., 2018). Although further production improvements are necessary, 2G biofuels offer significant advantages over 1G biofuels. The main advantage is that it limits the direct food versus fuel competition associated with 1G biofuels (Ojeda, *et al*., 2011). Feedstock necessary for this process can be bred specifically for energy purposes, which enables higher production per unit land area. This also means that a greater

amount of the above-ground plant material is converted and used to produce biofuels and as a result, this will further increase land-use efficiency compared to 1G biofuels (Ojeda, *et al*., 2011). Lignocellulosic materials are universally available in vast quantities, offer significant GHG savings, and have a very low, and in some cases, negative cost since many of the feedstocks belong to waste streams that are often difficult to dispose of and generally difficult to valorise economically (Claes, *et al*., 2020). Common features of the 2G bioethanol production process which hamper their widespread utilization include (i) the presence of inhibitory molecules because of the pretreatment of biomass, and (ii) the inhibitory nature of the desirable products, for example, ethanol and other by-products (Davison, *et al*., 2016). Overcoming these obstacles adds to the cost of the production process, lowering the economic feasibility of biofuels.

1.2.3. Bioethanol

Bioethanol is one of the most common and important liquid biofuels that can be produced from a variety of cheap substrates (Saini, *et al*., 2014). It is mostly used in the transportation sector, having a long history as an alternative fuel. After World War II, the use of bioethanol was neglected, due to the expense of its production when compared to petroleum-based fuel, until the oil crisis in the 1970s (Azhar, *et al*., 2017). The interest in using bioethanol has been increasing since the 1980s and is already being used as an alternative fuel in many countries. Unfortunately, thus far, cellulosic bioethanol has not been produced stably at a scale acceptable for commercial use. Despite the many pilot and demonstration plants, the former DuPont facility in Nevada, Iowa (USA) was the only one designed for cellulosic bioethanol production from corn stover using the *Zymomonas mobilis* strain jointly developed with the National Renewable Energy Laboratory (NREL), having a projected production capacity of 30 million gallons of fuel bioethanol per year. It was opened in October 2015 but unfortunately closed in late 2017 due to poor economic performance (Xia, *et al*., 2019).

Bioethanol is currently one of the most promising alternatives and offers several advantages over conventional transport fuels because of its desirable characteristics. These include high octane value and good combustion efficiency, broader flammability limits, higher flame speeds, and increased heats of vaporization (Hasunuma & Kondo, 2012). In contrast to petroleum fuel, bioethanol is less toxic, readily biodegradable, and produces fewer air-borne pollutants, helping to reduce CO_2 build-up by recycling CO_2 that is released when bioethanol is combusted as fuel

(Hasunuma & Kondo, 2012). Bioethanol is rapidly being adopted as a liquid transport fuel due to established production technologies as well as the previously mentioned relative compatibility with existing infrastructure (den Haan, 2018). The wide range of raw materials used in the manufacturing of bioethanol are conveniently classified into three main types: (i) sugars; (ii) starches, and (iii) cellulosic materials (Saini, *et al*., 2014). Sugars, which include but are not limited to cane or sweet sorghum juice and molasses can be used directly for ethanol production via fermentation. Starches, from corn, cassava, potatoes, and root crops, must first be hydrolysed to fermentable sugars by the action of enzymes. Cellulose must also be converted into monomeric sugars, most commonly achieved by the action of acids or cellulolytic enzymes (Saini, *et al*., 2014).

1.3. Lignocellulosic Biomass

Lignocellulosic biomass (LCB) is the most abundant renewable resource in nature and is derived from a variety of sources such as terrestrial and aquatic plants, including agricultural, forest residues, trees, and crops (Favaro, *et al*., 2019). Agricultural residues such as wheat straw, rice straw, bagasse, cotton stalk, and wheat bran are all rich in lignocellulose (Saini, *et al*., 2014). Other sources include animal, industrial and municipal waste, as well as unicellular and multicellular microorganisms such as microalgae and fungi. (Amoah, *et al*., 2019). While bioethanol from LCB is a promising fuel candidate in terms of resource abundance and environmental sustainability, the overall persisting high cost for 2G bioethanol production renders the process commercially non-profitable (Zabed, *et al*., 2016). The conversion efficiency and ethanol yield of biomass differ greatly due to the diverse composition of LCB, which is highly dependent on the raw material from which the feedstock is obtained (Zabed, *et al*., 2016; Favaro, *et al*., 2019). LCB (Figure 1.2) primarily contains cellulose, lignin, hemicellulose, and extractives (Saini, *et al*., 2014; Amoah, *et al*., 2019).

Cellulose is the main component of LCB, making up nearly 40 to 60% of its total dry weight depending on the plant species (Hasunuma & Kondo, 2012). It is found in nature almost exclusively in the cell wall of plants, however, it is also produced by tunicates, and a few bacteria (Lynd, *et al*., 2002). It is the primary product of photosynthesis in terrestrial environments, and the most abundant renewable polymer produced in the biosphere, with roughly 100 billion dry tons produced a year (Zhang, *et al*., 2006). Morphologically, it is a linear homo-polysaccharide consisting of between 500 and 15 000 anhydrous glucose units that are linked by β-1,4-glycosidic

bonds, with cellobiose being the smallest repetitive unit. These highly crystalline regions are separated by less ordered amorphous regions (Saini, *et al*., 2014) and are embedded in a hemicellulose, pectin, and lignin matrix (Valenzuela-Ortega & French, 2019), a structural arrangement that makes it resistant to saccharification by hydrolytic enzymes. This is usually alleviated by chemical and physicochemical pretreatment of the LCB, causing the cellulose to swell, thereby increasing its accessibility to saccharification enzymes (Hasunuma, *et al*., 2012).

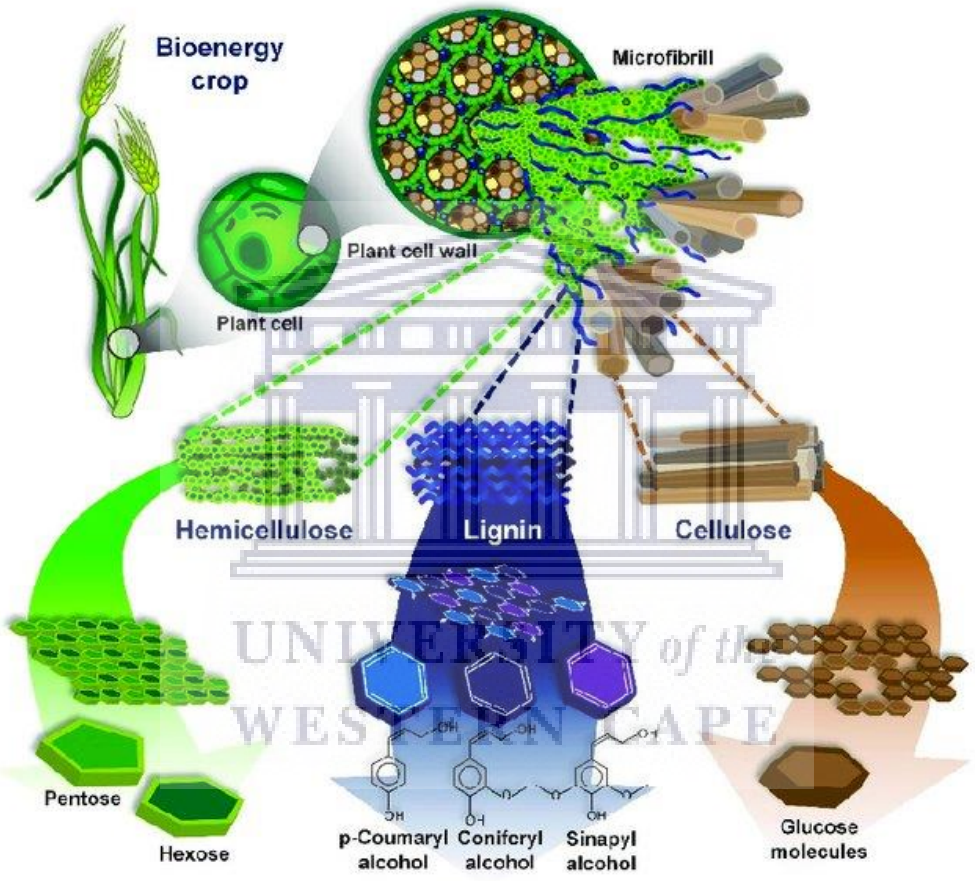

Figure 1.2: Diagrammatic illustration of the structure of lignocellulose and its biopolymers *(Hernandez-Beltrán, et al., 2019).*

Hemicellulose refers to several heterogeneous structures, such as xylan, galactomannan, and xyloglucan (van Zyl, *et al*., 2007). Xylan, the second most abundant polysaccharide found in LCB is more easily degraded into monomers by pretreatment processes than cellulose. For this reason, it has gained more attention as a source of sugar during the production of bioethanol (Liu, *et al*., 2016). Hemicelluloses are short, highly branched polymers consisting of around 50 to 200 units of pentose sugars, such as D-xylose and L-arabinose, and hexoses, such as D-mannose, D-galactose,

and D-glucose. Acetate groups are randomly attached with ester linkages to the hydroxyl groups of the sugar rings while hydrogen bonds link the hemicellulose molecules to the cellulose microfibrils (Hasunuma & Kondo, 2012; Saini, *et al*., 2014). The physiological role of hemicellulose is to provide a linkage between lignin and cellulose (Saini, *et al*., 2014). During most processes that involve the conversion of LCB to bioethanol, thermo-chemical hydrolysis of hemicellulose takes place first, followed by enzymatic hydrolysis of the cellulose part and a yeast-based fermentation of the resulting sugar monomers (Ollofsson, *et al*., 2008).

Lignin is present as an amorphous mass surrounding the cellulose and hemicellulose fibres (Hasunuma & Kondo, 2012). It is a heterogeneous and crosslinked aromatic polymer with trans-coniferyl, trans-sinapyl, and trans-*p*-coumaryl alcohols as the main aromatic components. Lignin is covalently bound to side groups on different hemicelluloses, forming a complex matrix that surrounds the cellulose microfibrils. The proportion of lignin within the plant cell wall varies from 2 to 40%. The presence of strong carbon-carbon (C–C) and ether (C–O–C) linkages in the lignin contributes to the strength of the plant cell wall as well as providing protection from attack by cellulolytic microorganisms (Saini, *et al*., 2014). This makes it extremely difficult to degrade, and while it could in principle serve as an excellent renewable source of aromatic compounds, such processes are not yet economically feasible, Currently, its only function in this process is in the necessity for pretreatment to disrupt its structure before hemicellulose and cellulose can be attacked (Valenzuela-Ortega & French, 2019).

1.3.1. Hydrolysis of Lignocellulosic Biomass to Ethanol

Despite its abundance, the recalcitrance and inaccessibility of LCB still pose major hurdles to efficient 2G bioethanol production (Pereira, *et al*., 2016). While biomass itself is considered cheap, the challenges associated with sourcing and transport as well as its pretreatment remains largely unsolved, even after decades of research. (Favaro, *et al.*, 2019). For most types of LCB, the enzymatic digestibility of cellulose is very low (<20%), which is why pretreatment to improve its accessibility is imperative (Zhang & Lynd, 2008). Pretreatment, however, is energy and capital intensive and sometimes requires the addition of inhibitory or toxic compounds such as acid catalysts in addition to the inhibitory compounds produced during the degradation of the biomass (Favaro, *et al.*, 2019). It is for this reason that the cost of the bioconversion of these materials to

ethanol is much higher than that of conventional sugar sources such as corn starch and cane juice (Inokuma, *et al*., 2014).

Pretreatment methods are divided into four main groups namely biological, physical, chemical, and physicochemical. Each method has its advantages and disadvantages, dependent on both the biomass source and the inhibitory products formed (Favaro, *et al*., 2019). For a pretreatment method to be regarded as successful it has to meet certain requirements, including creating simple sugars (or making hydrolysis easier), avoiding the degradation of carbohydrates during pretreatment, avoiding the formation of compounds inhibitory to hydrolysis and fermentation and finally it must be cost-effective. Dependent on the pretreatment method selected, the removal of different quantities of hemicelluloses occur, however, from an economic perspective, it is important that the sugars contained in the hemicellulose fraction of lignocellulose, including the xylose and arabinose, are also converted to ethanol (den Haan, *et al*., 2013). During conventional thermochemical pretreatment, the recalcitrance of lignin and cellulose are efficiently removed, however, this process also generates a series of carboxylate, furfural, and phenolic by-products which are toxic to fermentation microbes (Hasunuma, *et al*., 2012). Metabolic engineering is useful to help develop novel pathways for detoxifying these inhibitors. Numerous types of enzymes can transform the inhibitor molecules into less toxic or even useful products. An example of this is the ability of peroxidases to oxidize phenolic compounds. It may be feasible to express these enzymes in fermentative microbes to enhance their resistance to these toxic compounds (Liu, *et al*., 2018). The enzymatic saccharification stage following pretreatment incurs further costs due to the necessary addition of commercial enzymes, containing cellulases and xylanases (Zhang & Lynd, 2008; Lynd, *et al*., 2017).

Many microorganisms exist that can degrade LCB, especially in warm and wet conditions such as your typical fermentation setup, but do not do so in the timeframes preferred by industry (Kroukamp, *et al*., 2017). Furthermore, these microorganisms often do not produce desirable commodity products such as fuels or produce desirable products, but in quantities that would not suit commercial processes (Menon & Rao, 2012). Naturally cellulolytic organisms also tend to be highly susceptible to the pretreatment derived inhibitors. The ideal fermentative host strain for biological production of fuels and chemicals would hence need to both tolerate the complex and challenging fermentation medium presented by lignocellulosic hydrolysates, as well as display

high levels of cellulase activity to produce commodity products at a commercial scale (Davison, *et al*., 2016). However, engineering these microorganisms with this in mind has proven to be a difficult task thus far (Valenzuela-Ortega & French, 2019). The recalcitrance of LCB, development of appropriate pretreatments, production of appropriate hydrolytic enzymes, and the development of ethanologens able to thrive in the hostile fermentation environment are persisting challenges that need to be overcome for the growth and sustainability of the 2G biofuels industry (Kroukamp, *et al*., 2018).

1.4. Enzymes Required for Cellulose Hydrolysis

Acid hydrolysis or chemical and physical methods are the standard conventions when pretreating LCB to increase enzyme digestibility (Zhao, *et al*., 2009). This is followed by enzymatic hydrolysis which helps to open the plant fibers and convert the polymers of cellulose and hemicellulose to sugars that can subsequently be fermented. The saccharification process is highly dependent on the synergistic actions of different cellulase and hemicellulose enzymes whose joint action degrades lignocellulose (Ojeda, *et al*., 2011; Hasunuma, *et al*., 2012).

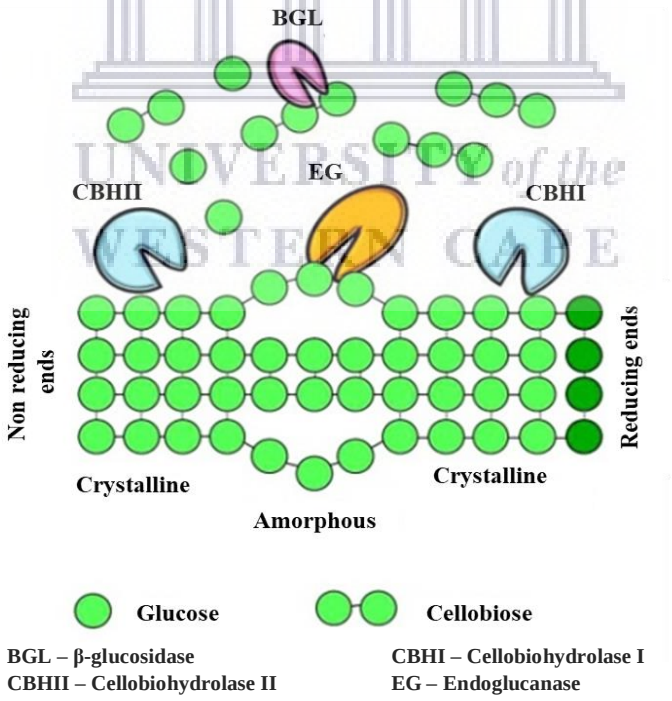

Figure 1.3: Diagrammatic representation of the enzymatic hydrolysis of cellulose by the synergistic action of cellulases *(Ratanakhanokchai, et al., 2013)*.

The cellulase complex is typically responsible for complete cellulose hydrolysis and consists of: endoglucanases (EG) that attack amorphous cellulose regions providing new chain ends, exoglucanases (cellobiohydrolases, CBH) that attach to chain ends and act in a processive manner, releasing cellobiose and β-glucosidases (BGL) that are responsible for cleaving cellobiose or oligosaccharide units into glucose monomers (Figure 1.3) (Yang, *et al*., 2016; Saini, *et al*., 2014).

To efficiently hydrolyse LCB into fermentable sugars, a large amount of these enzymes are needed (Amoah, *et al*., 2019). With currently available commercial enzymes, a kilogram of cellulose requires 20 g of cellulase to attain approximately 70% hydrolysis in five days. For the enzymatic hydrolysis to be more economically feasible, it is necessary to lower the amount of exogenous commercial cellulase required for this process. Since cellulase reactions are inhibited by their intermediary and final products, such as cello-oligosaccharides and glucose, combining enzymatic hydrolysis and sugar consumption steps would be preferential to prevent cellulase activity inhibition (van Zyl, *et al*., 2007).

1.4.1. Endoglucanase (EG)

Endo-β-(1,4)-glucanases (or 1,4-β-D-glucan-4-glucanohydrolases, EC 3.2.1.4), more commonly referred to as endoglucanases (EG), are characterized by their random hydrolysis of β-(1,4)-glucosidic linkages (Saini, *et al*., 2014). These β-1-4 glycosidic bonds are hydrolysed at random internal amorphous sites in the cellulose, to produce oligosaccharides in varying degrees of polymerization, thereby generating more chain ends and accelerating the recruitment of CBH (Liu, *et al*., 2016; Hasunuma, *et al*., 2012; Davison, *et al*., 2020). The function of EG in preferentially cleaving the amorphous regions of cellulose reduces the chance of CBH being unable to pass through this region, which would result in the stalling of CBH upon encountering an amorphous region (Liu, *et al*., 2017). This random cleavage of EGs causes a rapid decrease in chain length when acting on soluble cellulose derivatives and thereby changes in viscosity relative to the release of reducing end groups can be observed (Saini, *et al*., 2014). Additionally, in a free-form cellulase system, CBH can escape from being stuck by dissociating from the cellulose surface, however, in a cell-surface display system (discussed in section 6.1.2 below), the immobilized CBH will remain stuck to the cellulose. It is in a case such as this that more EG is required to clear the obstacles (Liu, *et al*., 2017).

1.4.2. Cellobiohydrolase (CBH)

Exoglucanases (Exo-β-(1-4)-glucanase or 1,4-β-D-glucan cellobiohydrolases, EC 3.2.1.91) are more commonly referred to as cellobiohydrolases or CBH (Saini, *et al*., 2014) and act on crystalline cellulose in a processive manner starting at the free chain ends (den Haan, *et al*., 2013; Liu, *et al*., 2016). These are further differentiated as CBHI and CBHII. The CBHI enzyme acts on the reducing end while CBHII acts on the non-reducing ends of the cellulose chains, releasing cellobiose as its major product (Davison, *et al*., 2020). It should be noted that CBHI and CBHII act synergistically during the hydrolysis of crystalline cellulose, and due to them hydrolysing the cellulose chain from different ends, co-expressing both these cellulases in a single strain has been shown to greatly improve the hydrolysis of crystalline cellulose (Ilmén, *et al*., 2011). These exoglucanases are also able to hydrolyse microcrystalline cellulose. It is postulated that this is achieved by peeling cellulose chains from the microcrystalline structure (Hasunuma, *et al*., 2012).

Due to their action in deconstructing crystalline cellulose, CBHI enzymes have been recognized as the most functional enzymes in fungal cellulase systems found in nature. For this reason, they have become the primary consideration to be expressed in yeasts for the creation of CBP microorganisms (see below) (Xu, *et al*., 2018). Their importance in both academic and industrial fields has led to these enzymes having served as one of the main cellulase proteins in numerous studies (Ilmén, *et al*., 2011; Sun, *et al*., 2018; van Zyl, *et al*., 2014; Davison, *et al*., 2019; Lamour, *et al*., 2019; Davison, *et al*., 2020). While the genes for this enzyme have been successfully expressed in yeast such as *Saccharomyces cerevisiae*, problems such as low yield of their recombinant proteins and low activities of those enzymes caused by low secretion and hyper-glycosylation, often affect their efficacy (Song, *et al*., 2018).

It has been reported that CBHII, on the other hand, moves in a less processive manner than CBHI with a study by Liu and co-workers (2017) finding that the level of CBHII needed for crystalline cellulose degradation was relatively low when compared to that of CBHI (Igarashi, *et al*., 2011). A previous study also by Liu and co-workers in 2016 showed that while *Talaromyces emersonii* CBHI appeared more important than *Chrysosporium lucknowense* CBHII in the synergism with BGL and EG, it was also found that the presence of both CBHI and CBHII in an enzyme cocktail was more effective in improving cellulolytic activities than increasing the proportion of CBHI. This is because CBHII can synergistically enhance the hydrolysis efficiency of CBHI by

diminishing the bumpy surface on cellulose which then prevents CBHI from getting stuck during processive movement (Liu, *et al*., 2016). Similarly, den Haan and co-workers (2013) showed that CBHII contributed to the opening of the structure of cellulose, allowing more CBHI to bind and hydrolyse the substrate. It was then further deduced that the endoglucanase II (EGII) cellulase (which is one of the most common endoglucanases) was, in fact, the largest contributing factor for successful synergism, which was highly efficient, even at exceptionally low dosages. Without CBHI and CBHII present EGII could release some cellobiose and glucose, likely due to repeated action on the released cello-oligomers. However, the release of glucose by this enzyme was shown to be significantly lower than that produced by the CBHs (den Haan, *et al*., 2013). This was consistent with observations on natural enzyme secretors, as the endo-exocellulase system of *Trichoderma. reesei* consists of around 60% CBHI, 20% CBHII and 12% EG (Zhang & Lynd, 2004). However, the optimum expression ratio of various cellulolytic enzymes for efficient cellulose degradation remains unknown, and the ratio is highly dependent on the content of the cellulosic material, which differs between sources and pretreatment method used (Yamada, *et al*., 2010).

1.4.3. β-glucosidase (BGL)

β-glucosidases (BGL or β-D-glucoside glucohydrolase, EC 3.2.1.21) play an important part in the cellulose degradation system because they are responsible for the final step of lignocellulose conversion. The BGL enzyme hydrolyses cellobiose, cellodextrin and other very short chain β-1,4-oligoglucosides up to cellohexaose, forming glucose in the process and is also involved in transglycosylated reactions of β-glycoside conjugates (Davison, *et al*., 2020; Saini, *et al*., 2014). Most BGLs are active against a range of β-dimers of glucose. Unlike exoglucosidases, the rate of hydrolysis of cellobiose will drastically decrease as the degree of polymerization of the substrate increases (Saini, *et al*., 2014). Furthermore, since cellobiose and cellodextrins are potent inhibitors of cellulose hydrolysis, a decrease in the cello-oligosaccharide amount leads to an increase in the saccharification rate by enabling cellulolytic enzymes to work more effectively (Smekenov, *et al*., 2020). Due to its efficacy in relieving the feedback inhibition of cellobiose overexpression, BGL has been reported to significantly improve the hydrolysis efficiency of cellulase mixtures (Liu & Qu, 2018).

1.5. Saccharification and Fermentation Bioprocesses

The biological processing of LCB involving enzymatic or microbial hydrolysis (Figure 1.4) commonly follows four biologically mediated processes: (i) the production of saccharolytic enzymes (cellulases and hemicellulases); (ii) the hydrolysis of carbohydrate components present in pretreated biomass to sugars; (iii) the fermentation of hexose sugars (glucose, mannose and galactose); and (iv) the fermentation of pentose sugars (xylose and arabinose) (Lynd, *et al*., 2005). Thereafter separation and concentration of ethanol by distillation and dehydration takes place to form the final product (Hasunuma & Kondo, 2012).

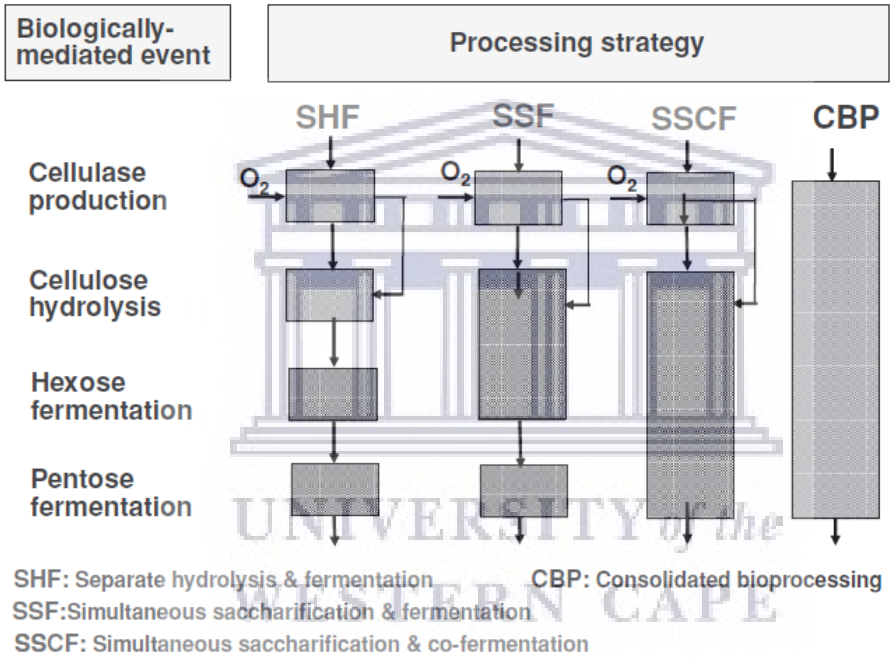

Figure 1.4: Common process configurations for the conversion of lignocellulosic biomass to bioethanol *(Zhang & Lynd, 2008)*

Enzymatic hydrolysis and fermentation may follow one of four different process configurations namely, Separate (or sequential) hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF), and finally CBP (Ojeda, *et al*., 2011).

1.5.1. Separate (Sequential) Hydrolysis and Fermentation (SHF)

The SHF is a two-stage process involving saccharification of the substrate which is followed by the separate fermentation of the saccharified fluid (Saini, *et al*., 2014). The main features of SHF

include optimal operating conditions regarding the pH and temperature for each step as well as minimal interactions between hydrolysis and fermentation steps (Ojeda, *et al.*, 2011). Due to the optimal conditions at which each step is performed, SHF allows for improved productivity rates of enzyme hydrolysis (Kroukamp, *et al.*, 2018). However, this process is limited by end-product inhibition, for example, glucose and cellobiose accumulation in the hydrolysis step inhibits the activity of the cellulases (Ojeda, *et al.*, 2011). The accumulation of monomeric sugars also leads to a higher risk of contamination, which in turn may decrease ethanol yields (Saini, *et al.*, 2014). Furthermore, since in SHF process each step is conducted separately, the enzymes that are produced within the process are difficult to recycle, thereby representing a loss from an economic standpoint (Oh & Jin, 2020). The external and continuous supply of theses enzymes adds to the costs which may cause challenges for industrial applications (Ollofsson, *et al.*, 2008).

1.5.2. Simultaneous Saccharification and Fermentation (SSF)

In SSF the hydrolysis of cellulose is merged with the direct fermentation of the glucose being released, alleviating the issue of product inhibition (Saini, *et al.*, 2014; Ojeda, *et al.*, 2011; Kroukamp, *et al.*, 2018). The avoidance of end-product inhibition on enzymatic saccharification results in higher ethanol yields and is most likely the key reason for using SSF, however, there are several additional advantages to using this process configuration (Ollofsson, *et al.*, 2008). This includes glucose not needing to be separated from the lignin fraction following a separate enzymatic hydrolysis step which avoids a potential loss of sugar. The SSF process also keeps the free sugar level continuously low, which counteracts bacterial contamination (Claes, *et al.*, 2020). Furthermore, the combination of hydrolysis and fermentation decreases the number of vessels needed, thereby decreasing investment costs (Saini, *et al.*, 2014). This decrease in capital investment has been estimated to be larger than 20% (Ollofsson, *et al.*, 2008). These advantages result in an increased rate of saccharification compared with SHF and total ethanol productivity in the SSF is notably higher than in the SHF process (Ojeda, *et al.*, 2011)

The principal drawbacks, on the other hand, are the different optimum conditions for enzyme hydrolysis and fermentation processes (Saini, *et al.*, 2014). The optimum temperature for enzymatic hydrolysis is typically higher than that of the fermentation step, at least when using yeast as the fermenting organism and consequently a need arises to find a favourable compromise with regards to the temperature and pH conditions. The high cost of exogenous enzymes that are

required during this process is another major factor to consider (Ilmén, *et al*., 2011; Ollofsson, *et al*., 2008). Additionally, difficulty of recycling the fermenting organism as well as the enzymes, due to the problems of separating the yeast from the lignin after fermentation poses a major economic problem. To satisfy the first requirement, the temperature is normally kept below 37°C, whereas the difficulty to recycle the yeast makes it beneficial to operate with a low yeast concentration and at a high solid loading (Ollofsson, *et al*., 2008).

1.5.3. Simultaneous Saccharification and Co-fermentation (SSCF)

The SSCF configuration is accomplished by combining the enzymatic hydrolysis of cellulose and the co-fermentation of pentose and hexose sugars into one reaction. This reduces the number of reaction vessels while also eliminating product inhibition (Ojeda, *et al*., 2011). Combining these two steps also reduces capital costs as well as processing time (Inokuma, *et al*., 2017). The biggest advantage of SSCF, however, is its reduced risk of contamination which is the result of almost no free sugars being accumulated during the process (Xia, *et al*., 2019).

In SSCF the co-fermenting microorganisms need to be compatible in terms of operating pH and temperature. However, there are not many microorganisms with the ability to ferment pentose sugars along with hexose sugars. The lack of an ideal co-fermenting microorganism (or consortium) is one of the greatest impediments in industrial production of 2G bioethanol via SSCF (Talebnia, *et al*., 2010). Using a microbial consortium may be a useful technology whereby a combination of hexose and pentose fermenting microorganisms is used for complete utilization of biomass sugars. An example of this is a study where the co-culturing of *Candida shehatae* and *S. cerevisiae* was reported as suitable for use in the SSCF process (Saini, *et al*., 2014). However, keeping consortia stable at industrial conditions is highly challenging due to varying pH and temperature optima and differences in nutrient requirements. Furthermore, catabolite repression still reduces pentose utilization in favour of glucose fermentation in these configurations.

1.6. Consolidated Bioprocessing (CBP)

In CBP, ethanol and all required enzymes are produced by a single microorganism or microbial community, in one reactor (Saini, *et al*., 2014). This process combines enzyme production, saccharification of polysaccharides, and fermentation of all available sugars in a single process. This approach has great potential for the cost-effective production of bioethanol from LCB, since

the costs of capital investment, substrates, and other raw materials, as well as utilities associated with microbial enzyme production are reduced by a substantial amount (Hasunuma & Kondo, 2012; Inokuma, *et al*., 2014). This process is however, only economically possible if microorganisms can be developed that possess the required combination of substrate utilization and product formation properties (Lynd, *et al*., 2002). The main advantage of CBP is that its application avoids the cost involved in the purchase or production of enzymes (Saini, *et al*., 2014). This approach is not only economically beneficial by replacing commercial enzymes but also simplifies the process in terms of operational units and offers the potential for higher efficiency compared to processes featuring dedicated cellulase production (Favaro, *et al*., 2019; Liu, *et al*., 2018). Beyond this, there are several factors supporting the possibility of realizing higher hydrolysis rates, leading to a reduced reactor volume and capital investment (Zhang & Lynd, 2008). Moreover, cellulose-adherent cellulolytic microorganisms are likely to successfully compete for products of cellulose hydrolysis with non-adhered microbes, which include contaminants (Lynd, *et al.*, 2005). Furthermore, these microorganisms are likely to be less sensitive to these contaminants, further increasing the stability of the industrial process (Zhang & Lynd, 2008). It is important to note that CBP is still currently a less efficient process with poor ethanol yields and longer fermentation time of more than 3–4 days. Nevertheless, significant cost reductions are expected when progressing from the previously mentioned processes to CBP (Saini, *et al*., 2014).

A microbial consortium displaying the desired capabilities may be one possible option to perform CBP. However, as previously stated, maintaining the optimal ratios of the microorganisms may prove too challenging for industrial applications (Oh & Jin, 2020). The use of a single microorganism is thus ideal, however, no natural microorganism with efficient enzyme production, lignocellulose saccharification, and ethanol production properties has yet to be identified (Inokuma, *et al*., 2014). While microorganisms producing all of the enzymes necessary for the degradation of the polysaccharides found in lignocellulose do exist, none display the ability to directly convert these to a commodity product, such as ethanol, at a rate or titre that would be considered economically desirable and those identified thus far have generally been shown to be highly sensitive to process derived inhibitors (la Grange, *et al*., 2010). Alternatively, microorganisms with beneficial product-producing qualities are often not able to hydrolyse polysaccharides, lack the ability to make use of all sugars available in biomass, or exhibit

sensitivity to the inhibitors present in pretreated LCB (den Haan, *et al*., 2013). Methods for the detoxification of these lignocellulose hydrolysates have been investigated and developed, but large-scale detoxification is a complex procedure and contributes to an increase in the fermentation process costs (Klinke, *et al*., 2004; Zhang, *et al*., 2010). Alternatively, engineering of CBP microorganisms must be undertaken that enable these microorganisms to thrive in the presence of a variety of toxic compounds that are produced during biomass pretreatment and found to inhibit the growth of the microbes, metabolism, and ethanol production (Hasunuma & Kondo, 2012; Klinke, *et al*., 2004). While this technology was suggested as a way to improve process economics almost twenty years ago, no organism with the required substrate conversion properties and biofuel productivity parameters has yet to be isolated or engineered (den Haan, 2018). There are several microorganisms, both bacteria and fungi, that possess some desirable properties for CBP that may form the basis from which process amenable strains can be engineered, and these microorganisms can broadly be divided into two groups: (i) native cellulolytic microorganisms that possess superior saccharolytic capabilities, but not desirable product formation, and (ii) recombinant cellulolytic microorganisms that naturally give high product yields, but into which saccharolytic systems need to be engineered (van Zyl, *et al*., 2007).

The native cellulolytic strategy involves improving ethanol production by engineering naturally cellulolytic microorganisms that lack efficient product formation (la Grange, *et al*., 2010). The crucial research objectives for this strategy include increasing ethanol titre, eliminating by-products, and improving the tolerance to ethanol (Hasunuma & Kondo, 2012). Thermophilic bacteria have attracted attention as CBP candidate organisms, due to being both cellulolytic and ethanologenic under thermophilic conditions (Rydzak, *et al*., 2009). Cellulolytic bacteria such as *Clostridium species* are promising CBP candidates but produce many unwanted by-products, including organic acids and nitrogen gas. *Clostridium thermocellum* is a Gram-positive, anaerobic, thermophilic bacterium and is a potential CBP organism due to its robust growth on crystalline cellulose (Lynd *et al.*, 2002). The intrinsic characteristics of *C. thermocellum*, however, limit its immediate and direct applications in bioethanol production from LCB as growth of natural *C. thermocellum* is slowed down by ethanol at concentrations greater than 2% (v/v), and 8% (v/v) for laboratory-evolved strains (Xu, *et al*., 2010). Extensive research has been carried out with model filamentous fungi such as *Neurospora crassa* and *Aspergillus nidulans*, as well as industrial fungi such as *A. niger, A. oryzae,* and *T. reesei* (Kunitake & Kobayashi, 2017). However, none of

these strains have been developed to a point where they can deliver high ethanol titres under 2G bioethanol processing conditions.

The recombinant cellulolytic strategy involves taking non-cellulolytic organisms exhibiting high product yields and engineering in a heterologous cellulase system that supports cellulose utilization (Hasunuma & Kondo, 2012). In this field, research focusses on improved secretion of the entire core set of cellulase enzymes required, improving strain robustness and broadening substrate utilization. The industry standard ethanologen yeast *S. cerevisiae* arguably remains the most promising CBP candidate and has been engineered to utilise the pentose sugar xylose and secrete cellulases (den Haan, 2018). However, low hydrolysis rates remain a major issue, which could be improved with the use of more digestible feedstocks in combination with improved cellulase activity. The cellulase activities of several CBP candidate organisms are notably lower than that of native cellulolytic fungi which is one of the several shortcomings that engineered CBP organisms display (den Haan, *et al*., 2015). For this reason, CBP using this method requires a highly engineered microbe developed to possess several different process-specific characteristics (Hasunuma & Kondo, 2012). Consequently, metabolic engineering of these microbes by heterologous gene expression and native gene deletion has become a research focus in this field (Anasontzis, *et al*., 2014).

1.7. Recombinant Cellulolytic Strategy: Engineering Yeast Strains

There are three major strategies for engineering yeast for the production of cellulases: (i) displaying enzymes on the cell surface (ii) secreting enzymes into the fermentation broth, or (iii) assembling and attaching artificial minicellulosomes onto the surface of the cell (Liu, *et al*., 2018; Favaro, *et al*., 2019; Liu, *et al*., 2015; Lynd, *et al*., 2005). Recombinant cellulases have been most often expressed as cell-surface displayed or secreted activities (Figure 1.5). Here the cellulose is hydrolysed extracellularly before glucose is transported into the cell and metabolized. Several combinations of genes such as *Saccharomycopsis fibuligera* β-glucosidase (Sf-BGLI), *T. reesei* endoglucanase (*Tr*-EGII) and *T. emersonii* cellobiohydrolase (*Te*-CBHI) have been expressed in yeast using these strategies and were shown to partially hydrolyse LCB (Davison, *et al*., 2019). Another strategy involves heterologous expression of a cellodextrin transporter and an intracellular β-glucosidase. In this strategy the cellodextrin transporter allows cellobiose and small cello-

oligosaccharides to enter the cell to be hydrolysed intracellularly to glucose by BGL which is then metabolized by the cell (Smekenov, *et al*., 2020).

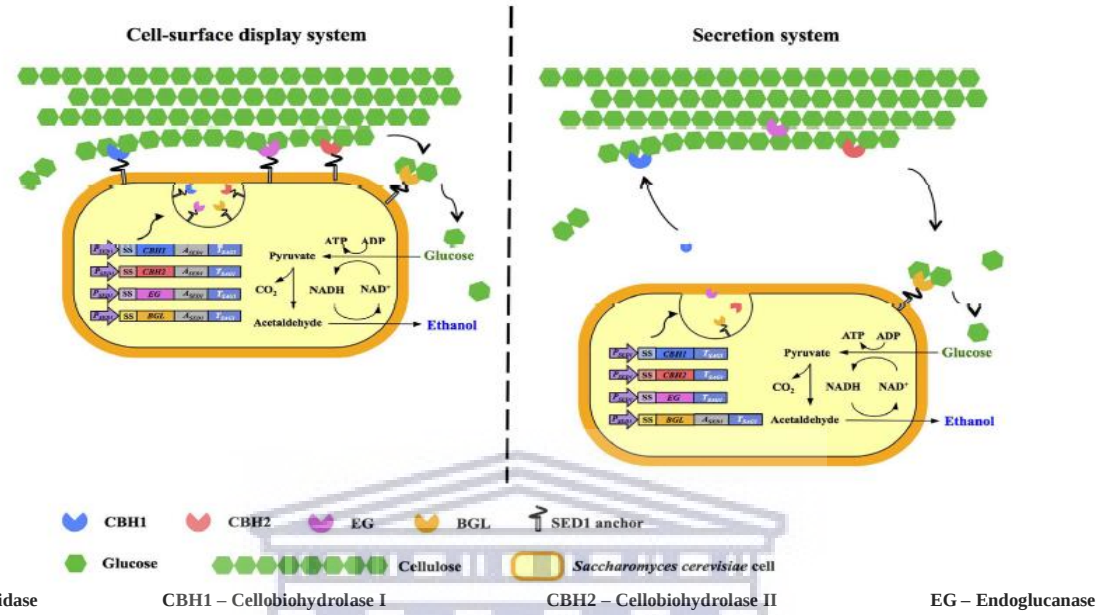

Figure 1.5: The two major strategies for engineering cellulolytic Saccharomyces cerevisiae for cellulosic ethanol production are via either cell-surface display or secretion of enzymes (Liu, *et al*., 2016)**.**

1.7.1. Secreted Cellulases

The most common route of cellulase production in recombinant strains is the cellulase secretion systems, in which enzymes are released into the extracellular environment (Liu, *et al*., 2016). This strategy is not limited by any physical restrictions, but only by the quantity of enzymes that can be secreted. Furthermore, due to the free nature of the cellulases, they can penetrate the secondary cell walls of plant biomass, thereby increasing cellulose accessibility. This was reported to be a critical factor in enzymatic hydrolysis (Liu, *et al*., 2015). It should, however, be noted that enzymes that diffuse away provide no direct benefit to the cells producing them, instead, benefitting the entire culture (den Haan, *et al*., 2015). Free enzymes also cannot be recycled during an industrial process (Liu, *et al*., 2016). To address these problems, *S. cerevisiae* strains displaying cellulases on their cell-surfaces were developed (Liu, *et al*., 2018).

1.7.2. Cell-Tethered Cellulases

An alternative to the secreted system is the engineering of yeast cells to display cellulolytic enzymes on the surface of the cell and is referred to as the cell surface display or cell-tethered system (Hasunuma & Kondo, 2012). This technology is based on the fusion of target peptides or

proteins with an anchoring protein motif containing a protein transport signal. This fusion protein can then be directed to yeast cell walls and displayed on the cell surface (Phienluphon, *et al*., 2019). Enzymes are displayed on the cell surface directly via an anchoring protein, such as SED1, a- or α-agglutinin, either directly or indirectly via cohesin-dockerin interactions to a surface-displayed scaffoldin to create a 'minicellulosome' (Valenzuela-Ortega & French, 2019). The use of *S. cerevisiae* for this system enables post-translational modification of heterologous eukaryotic proteins. Additionally, its rigid cell walls (around 110-200nm wide) as well as the constituents of the cell wall play a central role in anchoring cell-wall proteins. For this reason, *S. cerevisae* has become the preferred choice for this purpose (Liu, *et al*., 2016; Phienluphon, *et al*., 2019).

Cell tethering predominantly employs the glycosylphosphatidylinositol (GPI)-anchoring system to immobilize heterologous proteins on the cell surface (Inokuma, *et al*., 2020). In this method, fusion genes encoding the proteins of interest and the anchoring domain of the GPI-anchored cell wall protein (GPI-CWP) are transformed into the yeast cell. The GPI-CWPs are made up of three common domains: the N-terminal signal sequence for protein secretion, a serine/threonine (S/T)-rich region for glycosylation, and a GPI attachment (ω) site which is found near the C-terminal end of the protein (Phienluphon, *et al*., 2019). During secretion, the GPI-attached proteins leave the endoplasmic reticulum (ER) via coat protein complex II (COPII) coated vesicles where they then travel through the Golgi to the plasma membrane. Thereafter, the proteins are released from the plasma membrane and are immobilized in the cell wall via covalent linkage to a β-(1-6) glucan via the anchoring structure (Inokuma, *et al*., 2020). This anchoring system has allowed for various functional proteins to be displayed on the cell surface without affecting the activity of the proteins (Oh & Jin, 2020).

It has been reported that the efficiency of target enzymes used for cell-surface display via anchoring domains differ across different GPI-CWPs (Oh & Jin, 2020). For this reason, the appropriate anchoring domain for fusion with target proteins must be considered for efficient cell-surface display. Earlier work in this field reported a *S. cerevisiae* engineered with α-agglutinin fusion genes to display *T. reesei* EGII and *A. aculeatus* BGLI. The co-display of these genes allowed the recombinant strain to directly ferment phosphoric acid swollen cellulose (PASC) and an immediate increase in ethanol production was seen once the fermentation process began, yielding up to 88.5% of the theoretical maximum within 40 hours (Liu, *et al*., 2015). While these

results out-performed the EG- and CBH-secreted strains by 67.4%, some cellulolytic enzymes fused with the SED1-anchoring domain (a stress-induced structural GPI protein) were found to have even higher activities than those fused with the α-agglutinin (SAG1)-anchoring domain (Inokuma, *et al*., 2014). The degree to which activity was improved by a change in the anchoring domain therefore depended greatly on the enzymes displayed (Inokuma, *et al*., 2020).

Studies comparing surface display to the secretion method often report a slight advantage for the cell surface display method, especially where multiple enzymes are displayed on a single cell rather than using a consortium of microbes, each displaying one enzyme (Valenzuela-Ortega & French, 2019). Continuing with the comparison between the two technologies, it was noted that immobilization of enzymes on the cell surface increased their effective concentration, and this proximity could have a synergistic effect on cellulose-degrading activity (den Haan, *et al*., 2015; Oh & Jin, 2020). The synergetic effects displayed by EG, CBH and BGL as well as the optimization of the ratios are necessary for the effective hydrolysation of cellulose (Inokuma, *et al*., 2014). Another way to reinforce the synergism among cellulases is to increase the diversity of the cellulase species (Liu, *et al*., 2016), with a number of studies demonstrating that this yielded an increase in ethanol production while also rationally distributing intracellular resources for protein synthesis (Davison, *et al*., 2020; Inokuma, *et al*., 2020; Chen, *et al*., 2018).

Other advantages of the cell-surface display method include glucose being liberated from cellulose in close proximity to the cell surface, where it is immediately taken up, thereby minimizing the risk of contamination or product inhibition (Liu, *et al*., 2016). This also diminishes the repression effects of substrates on the microorganism. Long-distance mass transfer of the substrate is prevented by significantly shortening the distance from enzyme-to-enzyme, especially in high-solid fermentation. This method also alleviates the irreversible adsorption of cellulases on the crystalline cellulose (non-productive binding), resulting in enhanced ethanol yields (Inokuma, *et al*., 2014; Oh & Jin, 2020). Additionally, the production and auto-immobilization of proteins occur via easy cell propagation, which can reduce the expenditure and facilities needed for protein generation and enrichment (Liu, *et al*., 2015).

During fermentation, strains engineered using this method resulted in the enzymes being retained for as long as the yeast continues growing. This method therefore outperforms the secretion method, where it is not easy to maintain the activities for a long reaction period (Hasunuma &

Kondo, 2012). Due to the proteins being anchored on solid surfaces, they are considered more stable than free-form proteins under extreme conditions, for example, high temperature and in the presence of organic solvents, as is the case during fermentation processes. This stability will facilitate long-term storage and recycling in industrial processes especially since proteins can be easily recollected through centrifugation or filtration (Liu, *et al*., 2016). While there are many upsides to using the cell surface display method, inefficiency of processive enzymes, such as CBH has been reported, resulting in decreased crystalline cellulose conversion efficiency compared to free enzyme systems (Liu, *et al*., 2015; den Haan, *et al*., 2015).

Inokuma and co-workers (2020) recently demonstrated a refinement of the technique by controlling the localization of cellulases on the cell surface. In this study, EGII was localized to the external surface of the cell wall using the SED1-anchoring domain. This was necessary as EG requires contact with bulky insoluble cellulose in the media. BGLI on the other hand was immobilized on the inside of the cell wall though fusion with the SAG1-anchoring domain. As a result, competition for space on the outer surface of the cell was avoided. Results indicated that the strain containing the BGLI on the inside and EGII on the outside of the cell wall outperformed the strain in which both cellulases were displayed on the outer surface by producing a higher ethanol yield from a cellulosic substrate. These results indicated the importance of the anchorage position of target proteins in yeast cell surface display systems (Inokuma, *et al*., 2020).

2.1.1.1. δ-Integration

δ-sequences are dispersed throughout the yeast genome and are present in more than 200 copies, making them a good target for multiple and simultaneous integrations of genes at multiple sites. The δ-integration method is a technique that allows multicopy gene-integration in yeast by targeting these δ-sequences for simultaneous integration of cellulases (Yamada, *et al*., 2010). Initial studies had hoped that utilization of a cellulases cocktail was expected to increase the decomposition of lignocellulose, however it was found that maintaining the expression of several cellulase genes at an identical level was a difficult task. The ratios of cellulases in naturally cellulolytic organisms are adjusted as required by the regulation of the expression of the cellulase-encoding genes. This takes place by addressing the issues brought about by chemical heterogeneity and the structural complexity of natural lignocellulosic substrates. It is therefore necessary to optimize the enzyme ratios for each specific substrate used (Davison, *et al*., 2020). With cocktail δ-integration, the use of a single step allowed for several kinds of cellulase expression cassettes to

be integrated into yeast chromosomes simultaneously, at different copy numbers. Using this integration method, a strain expressing cellulase ratios ideal for growth on PASC was created (Yamada, *et al*., 2010). The genetic properties of plasmids used for this method are illustrated in Figure 1.6.

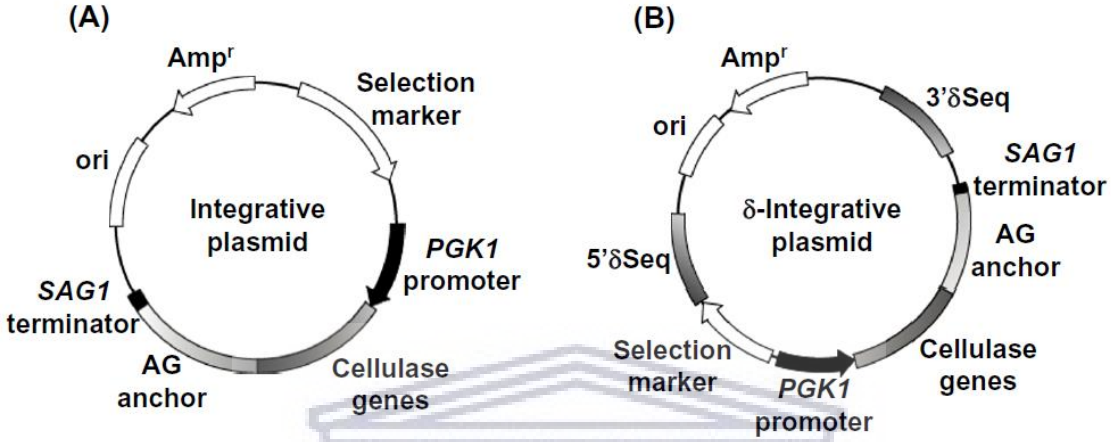

Figure 1.6: Schematic representations of cellulase expressing plasmids. (A) Plasmid for single copy integration. (B) Plasmid for δ-integration (Yamada, *et al*., 2010)**.**

δ-Integration vectors are also good at achieving stability and copy number improvements (Liu, *et al*., 2018). This is because integration takes place on multiple chromosomes at the same time due to the presence of approximately 425 δ-regions dispersed throughout the yeast genome (Liu, *et al*., 2017; Sasaki, *et al*., 2019).

A strain engineered by Yamada and co-workers (2011), using cocktail δ-integration, was able to retain 16 copies of EGII, 6 copies of CBHII, and 1 copy of BGLI and produced 3.1g/L ethanol from 20g/L PASC. Using δ-integration, several research groups also improved the cellulosic hydrolysing and fermenting capacity of *S. cerevisiae* strains (Liu, *et al*., 2017; Song, *et al*., 2018; Davison, *et al*., 2019). Liu and co-workers (2017) screened for transformants using crystalline cellulose as the substrate. The ethanol yield from avicel generated by the resultant cellulolytic yeast produced by this technique was the highest ever reported for crystalline cellulose at 2.9g/L, while the control strain was only able to produce an ethanol concentration of 1.9g/L (Liu, *et al*., 2017). It is these strains that we will seek to further improve through strain engineering in the current study.

1.8. *Saccharomyces cerevisiae* – Industrial and Laboratory Workhorse

The yeast *S. cerevisiae* is a model eukaryote, best known for its large-scale use in the food industry for the production of bread and alcoholic beverages (Valenzuela-Ortega & French, 2019). It is the most used microorganism for industrial ethanol production and has attracted increasing interest for metabolic engineering, especially for processes that require scaling up for production of commercial products, making it an ideal candidate for industrial production of bioethanol and other commodity products (Inokuma, *et al*., 2014; den Haan, *et al*., 2015; Kroukamp, *et al*., 2018; Liu & Ma, 2020). Due to its long association with the food and beverage industries, *S. cerevisiae* is also classified under the category of generally regarded as safe (GRAS). Moreover, gene recombination methods have been well-established in *S. cerevisiae* resulting in the heterologous production of cellulases using this yeast (Mitsui, *et al*., 2019).

The numerous stress factors that are introduced during the fermentation process often reinforce one another and make it even more difficult to obtain the level of fermentation performance required under true industrial conditions (Deparis, *et al*., 2017). The use of laboratory yeast strains have been employed for most of the research conducted, however, strains such as these may perform inadequately in an industrial setting due to their overall weaker resistance to the stress factors present during these fermentations, as well as their poorer fermentative abilities when compared to common industrial strains (Smekenov, *et al*., 2020). Industrial strains also show higher ethanol productivity and yield, thermostability and higher tolerance to acids, ethanol and sugar than laboratory strains (Favaro, *et al*., 2015).

1.9. Potential of *S. cerevisiae* as a CBP organism

The physiological conditions in which 2G bioethanol is produced offers an environment that is far from the optimal for yeast (Favaro, *et al*., 2019). There are several stresses which microorganisms are subjected to throughout the process of bioethanol production. The most relevant of these stresses are the high sugar and ethanol concentrations, oxidative stress, elevated temperatures, intensive cell recycling and pH variations. It is for these reasons that a microorganism capable of thriving in these unfavourable conditions, without changing the fermentative conditions must be selected. For application in CBP, a microorganism that is not only robust but also has a high secretion titre as well as the ability to use substrates under anaerobic conditions would be an ideal

fit for this process (Olson, *et al*., 2012). Many researchers have reported improvements in yeast tolerances using techniques such as genetic engineering, adaptation, ultraviolet (UV) and chemical mutagenesis, protoplast fusion, and gene shuffling (Steensels, *et al*., 2014). These methods have made substantial contributions in improving the tolerances of industrial yeasts for several decades (Inokuma, *et al*., 2017). The advantages and disadvantages of using *S. cerevisiae* as a potential CBP organism are listed in Table 1.1, showing several key areas where research is required to improve this organism to industrial standard.

It has not yet been determined how many lignocellulolytic enzymes can be successfully expressed simultaneously in a 2G industrial yeast strain and whether or not the increased number of enzymes added would negatively impact other pathways such as xylose utilization that may have already been artificially engineered in these strains. However, Claes and colleagues (2020) recently showed that by using the CRISPR/Cas9 technology they were able to create the first 2G xylose utilizing industrial *S. cerevisiae* strain that simultaneously expressed and secreted seven cellulolytic enzymes. This engineered strain allowed for direct ethanol production from multiple lignocellulosic substrates, using both the glucose and xylose sugar components (Claes, *et al*., 2020).

1.10. Problems Associated with Heterologous Cellulase Production

Den Haan and co-workers (2013) reviewed reports of several strains of *S. cerevisiae* that were created to convert cellobiose, amorphous cellulose and crystalline cellulose to ethanol. These reported on the secretion of CBH by *S. cerevisiae* and other yeasts which were subsequently tested for increased activity on a variety of substrates ranging from small synthetic molecules to amorphous and crystalline forms of cellulose. These studies reported low levels of secreted CBHs as a common feature of heterologous CBH production in yeast, although the range of reported values was quite large. This, together with the low specific activity of CBHs on polymeric substrates in yeasts led to the identification of CBH expression as a limiting factor for CBP (den Haan, *et al*., 2013).

Table 1.1: Advantages and disadvantages of Saccharomyces cerevisiae as a potential CBP organism.

Advantages	Disadvantages
High rate of fermentation, especially for industrial strains (Deparis, *et al*., 2017)	Innately low level of protein secretion (Ilmén, *et al*., 2011).
High propagation rate, especially for industrial strains (Deparis, *et al*., 2017)	Pretreatment inevitably releases phenolics, furans, and organic acids that inhibit yeast performance (Almeida *et al.*, 2007)
High overall robustness (Deparis, *et al*., 2017). Due to its process robustness, it is known to have a higher tolerance to ethanol (> 10% v/v) and other compounds present in lignocellulosic hydrolysates due to its ability to acquire adaptive resistance to stress conditions	The chemicals for which a microorganism is usually exploited, such as organic acids or alcohols, exert strong inhibition on the metabolism of these microorganisms (Deparis, *et al*., 2017)
Significant progress has been made in engineering strains of *S. cerevisiae* for cellulose CBP including successful production of the three main types of cellulases namely, BGL, EG, and CBH and partial conversion of crystalline cellulose to ethanol was shown (Liu, *et al*., 2015).	Temperature gradients introduced during the fermentation process and fluctuations in the fermenters can negatively impact the fermentation rate and productivity of the yeast (Deparis, *et al*., 2017).
Can natively use glucose, as well as maltose, isomaltose, sucrose, and some other saccharides (Valenzuela-Ortega & French, 2019)	Unable to utilize cellobiose, xylose, or arabinose (Valenzuela-Ortega & French, 2019)
Able to grow well in anaerobic conditions such as in a fermentation vessel (Inokuma, *et al*., 2014)	Regardless of their robustness, industrial yeast often lacks tolerance to specific stress factors when used as cell factories (Deparis, *et al*., 2017)
Provides a higher ethanol yield from glucose in comparison to other bacteria (Deparis, *et al*., 2017)	
Demonstrates simple handling in inexpensive media formulations, whilst simultaneously being devoid of pyrogens, pathogens, or viral inclusions (van Zyl, *et al*., 2014)	
Compared to almost all bacteria, it has a more rapid sugar consumption (Kroukamp, *et al*., 2018)	

As previously mentioned (section 1.4) the complete hydrolysis of cellulose to glucose requires a combination of BGL, CBH and EG cellulase enzymes which work in a synergistic manner for the hydrolysis of both native and modified cellulose (Saini, *et al*., 2014). The challenge with this is to develop recombinant cellulolytic microorganisms that can express high levels of these cellulases to support cell growth on crystalline cellulose (Zhang & Lynd, 2008). Unfortunately, heterologous production of these in *S. cerevisiae* at the required high levels has proven problematic thus far (van Zyl, *et al*., 2007). While relatively high-level expression of EGs and BGLs were shown, reported CBH secretion levels have been poor (den Haan, *et al*., 2013). A paper published by Ilmen and co-

workers (2011) reported successful expression of various cellulases and observed that some candidates were more compatible with yeast expression than others. Furthermore, a study conducted by Yamada and co-workers (2010) demonstrated that producing cellulase activities in the correct ratios improved PASC hydrolysis rather than simply greater overexpression of the cellulase encoding genes. Using this information, we are now able to select candidate cellulases representing the three required types that express well in *S. cerevisiae*, test their apparent synergistic action, and apply this knowledge in the construction of cellulose-degrading strains of this yeast (den Haan, *et al*., 2013).

Several lines of research are aimed at enhancing heterologous enzyme production, as well as their activities (den Haan, *et al.*, 2015). It has been speculated that low heterologous cellulase secretion from *S. cerevisiae* is due to several secretory bottlenecks that form because of overproduction and improper folding of recombinant proteins. Reports suggest that heterologous proteins stimulate the unfolded protein response (UPR) which are then degraded due to the induction of the endoplasmic reticulum-associated degradation machinery (ERAD) (Davison, *et al*., 2016). Appropriate strain engineering has been investigated to alleviate this problem.

1.11. Strain Engineering for Enhanced Expression of Cellulases

Discovery and understanding of molecular biology and genetic engineering have led to an increased interest in yeast as an expression host for native and recombinant proteins used in the biopharmaceutical, agricultural and enzyme industries (Kroukamp, *et al*., 2018). By using genetic and metabolic engineering technologies, the desired genes could be overexpressed, and genes responsible for unwanted product formation could be blocked or deleted (Liu, *et al.*, 2018). Strain engineering for protein secretion generally follows four strategies: (i) engineering of protein folding and quality control (QC) system in the ER, (ii) engineering of the intracellular protein trafficking, (iii) minimization of post-secretory proteolytic degradation, and (iv) engineering of post-translational glycosylation (Idris, *et al*., 2010). The use of molecular tools, including high copy vectors, strong constitutive promoters, codon optimization and efficient secretion signals have all been well characterized in *S. cerevisiae* (Kroukamp, *et al*., 2017).

Before being released from the cell as a mature product, nascent proteins must progressively move through various intracellular compartments. This process can be made more efficient with the help of genetic engineering by relieving known bottlenecks at key points of the pathway (Kroukamp,

et al., 2018). However, a clear understanding of the changes and complexities brought about by gene alterations, the limitations involved, in addition to its driving mechanisms must be first uncovered. Strain engineering has led to substantial improvements in protein yields, however, its widespread use is hindered by the limited information on specific secretion factors and the protein-specific nature of these improvements (Kroukamp, *et al*., 2017). We will now briefly overview the yeast secretory pathway to illustrate how rational engineering can be used to improve cellulase secretion in *S. cerevisiae*.

1.11.1.Protein Secretory Pathway

The protein secretion pathway is the multi-step route which a nascent polypeptide of *S. cerevisiae* will follow, travelling towards the extracellular space, cell wall, plasma membrane, or organelles, and is often facilitated by transport through a bi-layered lipid vesicle, all of which constitute a highly complex system (Kroukamp, *et al*., 2018; Celińska & Nicaud, 2019). The continued discovery and understanding of this pathway and all of its complexities has allowed directed engineering approaches to improve the rate of protein secretion (den Haan, *et al*., 2013). Due to the many interacting participants, with each step being directed and regulated by numerous proteins, a wide variety of targets are available for exploitation to enhance heterologous protein secretion (Song, *et al*., 2018). Several of the proteins involved in the secretory pathway serve as sensors and regulators of QC points and due to this interdependency, all of these steps within the pathway must be synchronised seamlessly for smooth processing of protein traffic. Proper post-translational modification and folding are essential for cell viability (Celińska & Nicaud, 2019).

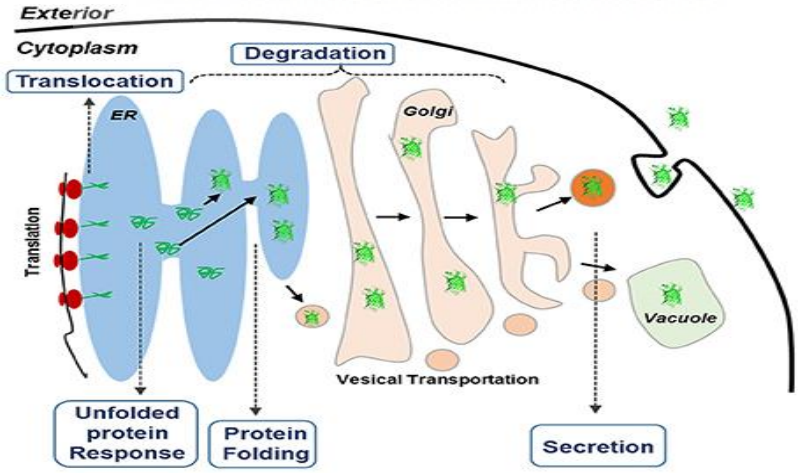

Figure 1.7: Schematic overview of the secretory pathways in yeast *(Sheng, et al., 2017)*

The protein secretory pathway (Figure 1.7) is initiated by one of two paths, either the co- or post-translational translocation, which occurs through the *SEC61* translocon into the crowded environment of the ER lumen (Idris, *et al.*, 2010). This step is found to be a potential bottleneck during recombinant protein secretion in both *Pichia pastoris* and *S. cerevisiae*. Often it is found that these nascent proteins will accumulate before translocation, creating an accumulation in the translocation channel.

The ER-resident protein folding and the QC system mainly involves five components: (i) molecular chaperones (e.g., BiP, calnexin, and calreticulin) that assist protein folding, (ii) enzymes such as protein disulphide isomerases (PDIs) and *cis-trans* peptidyl-prolyl isomerases (PPIs), (iii) degradation machinery linked with ERAD, (iv) signal transduction pathways linked with UPR, and (v) post-translational modification enzymes related to glycosylation (Figure 1.8).

Once translocated into the ER, nascent polypeptides are mediated by the ER-resident chaperone protein-binding protein (BiP; encoded by *KAR2*) for folding to native structures, whereas the nascent glycoproteins are bound by the ER chaperone calnexin (encoded by *CNE1*) to undergo their correct folding and *N*-glycan processing (Idris, *et al.*, 2010). The ER sustains a set of covalent modifications, which include signal sequence processing, *N*-glycosylation, disulphide bond formation, glycosyl-phosphatidyl-inositol addition, degradation, and sorting (Idris, *et al.*, 2010). The formation of correct disulphide bonds is mediated in a cycle of PDI and ERO1 activity, which may lead to the formation of reactive oxygen species (ROS) (Gasser, *et al*., 2008). Due to the ER maintaining this strict QC system, protein folding becomes the most rate-limiting bottleneck to overcome in heterologous protein secretion (Idris, *et al*., 2010). Overexpression by rational engineering strategies of core folding and translocation aiding factors (such as the *SSO1* and *SEB1* genes) have shown improved secretion levels of recombinant proteins in *Kluyveromyces lactis* (Idris, *et al*., 2010). Prolonged BiP binding, indicating misfolding or aggregation of nascent proteins are recognised by the QC system, leading to retrograde translocation to the cytosol which leads to the induction of the UPR, mediated by IRE1 which stimulates proteolysis by ERAD, inhibiting the transcription and translocation of the target protein (Idris, *et al*., 2010; Gasser, *et al*., 2008). Accumulation of misfolded proteins in the ER stimulates a rapid cellular response to relieve this stress or enter a programmed cell death.

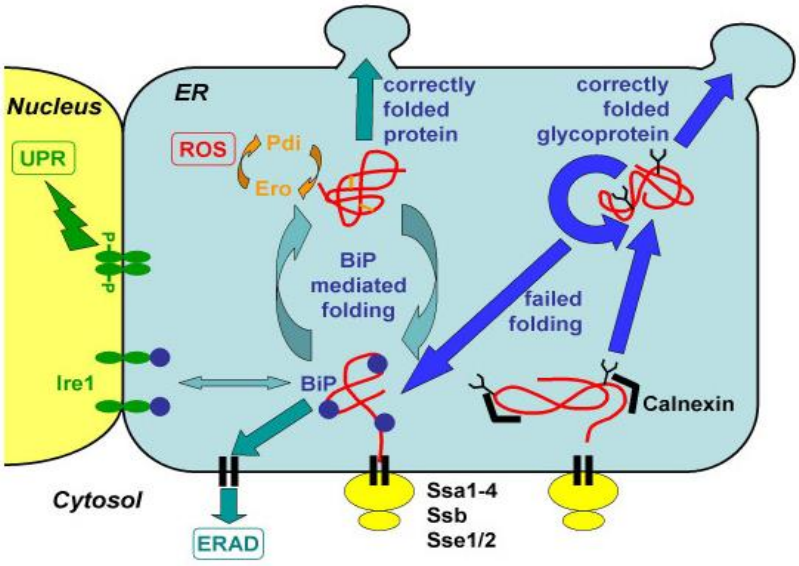

Figure 1.8:Schematic representation of protein folding, quality control, degradation and secretion in yeast *(Gasser, et al., 2008).*

Following the folding and modification processes in the ER, correctly folded proteins are then released to transport vesicles. The selective trafficking of secretory proteins between organelles in the secretory pathways starts by transporting them by means of these 40–60nm COPII vesicles to the Golgi apparatus to be further modified, and then transported to the extracellular space, vacuoles or other organelles (Idris, *et al*., 2010; Davison, *et al*., 2020). They are then finally transported to the plasma membrane in 80–100nm vesicles to the tip of the emerging bud (Kroukamp, *et al*., 2018).

The process of directing each newly expressed protein to the cell surface (referred to as protein targeting, or protein sorting) is crucial to the organization and functioning of yeast cells (Idris, *et al*., 2010). The effects of each trafficking step and correct vesicular destination at each crossroads are controlled by numerous intracellular membrane proteins that significantly affect the overall secretory effects. Thus, genetic optimization of trafficking pathways is required particularly in the case of inefficient trafficking or missorting (Idris, *et al*., 2010). Through genetic engineering, protein trafficking can be made more efficient, by relieving bottlenecks at key points of the pathway. Overexpressing components of vesicle formation, including components of the SNARE complex, has proven to be a positive means to improve the rate of transport (van Zyl, *et al*., 2014; van Zyl, *et al*., 2016; Davison, *et al*., 2020).

There have been numerous strategies investigated to increase the protein product available for secretion in *S. cerevisiae*. These include but are not limited to: (i) engineering of the peptide leader sequence, (ii) optimization of the gene copy number, (Ilmén, *et al*., 2011) (iii) manipulation of promoter strength, and (iv) engineering of the heterologous protein or gene of interest including codon bias optimization (den Haan, *et al*., 2013). These approaches have been combined with non-rational strategies, such as fermentation optimization and strain breeding approaches or rational secretory pathway optimization strategies to circumvent protein processing and transit bottlenecks. Examples of significant improvements through rational design include (i) increasing ER-resident chaperones and foldases, (ii) accelerating vesicle fusion events by overexpressing SNARE-complex proteins, (iii) altering the degree of protein glycosylation, (iv) modulating cellular stress, and (v) reducing intra- and extracellular proteolytic-loss of product (Idris, *et al*., 2010; van Zyl, *et al*., 2016; Kroukamp, *et al*., 2018). However, the majority of these improvements are protein-specific and require even further investigation (van Zyl, *et al*., 2016; Kroukamp, *et al*., 2013; van Zyl, *et al*., 2014).

1.11.2. Protein Secretion Stressors

Previously, it has been suggested that high levels of engineered secreted enzymes would result in metabolic stress and protein burden (Claes, *et al*., 2020). Pathways affected by this are responsible for folding, protein modifications and QC, such as ERAD and UPR (Figure 1.9) which help avoid the accumulation of unfolded proteins and intracellular transport through the cell. Once these pathways are affected the fermentation capacity of the host yeast strain becomes compromised (Claes, *et al*., 2020; Kroukamp, *et al*., 2018). They can, however, increase the folding capacity of the ER as well as remove misfolded or irregular proteins from the secretory pathway, so the dysfunctional proteins are not secreted under conditions of ER stress (den Haan, *et al*., 2013).

Excessive production of a given secretory protein inevitably leads to overloading the secretory pathway, accumulation of unfolded protein, and physiological stress. In addition to this, engineering novel metabolic pathways can also cause the added stress of redox imbalances (Deparis, *et al*., 2017; Celińska & Nicaud, 2019). The inability of the foreign polypeptides to reach their native conformation in heterologous host cells often leads to an abundance of these recombinant proteins being present in the insoluble cell fraction (Gasser, *et al*., 2008). It was demonstrated that excessive overproduction of heterologous enzymes, while leading to higher secretion of the polypeptides, caused a decrease in the overall specific activity of the secreted

enzymes. Excessive protein production could therefore negatively affect protein folding due to deficiency in chaperones and saturation of the secretion machinery (Dulermo, *et al.*, 2017).

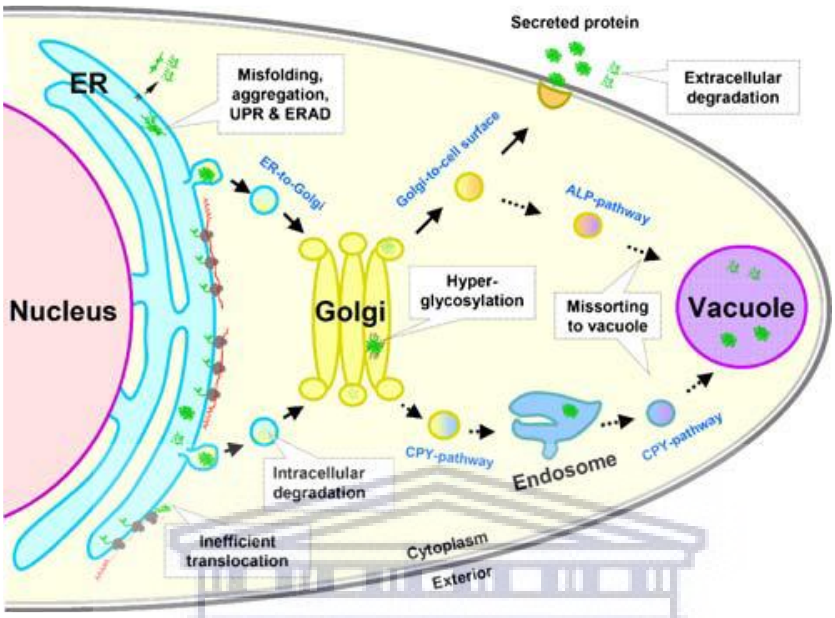

Figure 1.9: A diagram representing typical bottlenecks in the secretory pathway of heterologous proteins in yeast. The main membrane trafficking and vacuolar protein sorting pathways are indicated *(Idris, et al., 2010).*

Regarding heterologous cellulase expression, a study performed by Cedras (2020), showed that it is reasonable to assume that low secretion titres were in part a result of the burden that producing CBH caused on the secretory pathway. This problem is further magnified by the post-secretory (or extracellular) degradation of the recombinant gene products by host-specific proteases, which are present in relatively high levels in yeast, and are induced readily by environmental stresses, especially during high-density fermentation processes (Idris, *et al.*, 2010). The significance of this problem increases during secretory production of proteolytically sensitive heterologous proteins. Solutions to this issue have been investigated such as control of cultivation conditions, changing medium composition, and addition of protease inhibitors, peptone, casamino acids or specific amino acids to the fermentation media. Interestingly, a study performed by Claes and co-workers (2020) showed that robust 2G industrial yeast strains might tolerate much higher levels and numbers of secreted enzymes compared to the laboratory or 1G industrial yeast strains, possibly due to better innate stress modulation in this strain background.

1.11.3.Potential Gene Targets for Improved Heterologous Secretion

The need for further improvement with regards to the complex interactions between molecules during the production of enzymes and increased cellulase activity led to the discovery of key genes involved in regulatory networks responsible for enhanced protein translocation and secretion (Kroukamp, *et al*., 2018). Eukaryotic cells respond to the accumulation of unfolded proteins in the ER by activating their UPR, which is regulated by HAC1. Due to UPR induction, the expression levels of *KAR2*, *ERO1*, *PDI1* and *DER1* also increase (den Haan, *et al*., 2013; Song, *et al*., 2018). It was reported by Idris and co-workers (2010) that heterologous overexpression of *T. reese*i *HAC1* in *S. cerevisiae* yielded a 2.4-fold improvement in *Bacillus* α-amylase secretion. A 2-fold increase in endogenous invertase as well as a 70% increase in recombinant α-amylase was also noted. However, the secretion *T. reesei* endoglucanase EGI was unaffected (Idris, *et al*., 2010).

Overexpression of *YHB1*, encoding a nitric oxide oxidoreductase, as well as *SET5*, a histone H4 methyltransferase encoding gene was reported to increase tolerance to multiple fermentation inhibitors, for example, improved heat (*SET5*) and osmotic (*YHB1*) tolerance, while also improving heterologous CBH secretion (Lamour, *et al*., 2019). A decrease in the UPR was also observed, suggesting novel mechanisms for enhancing enzyme production through stress modulation. Furthermore, *SET5* has been found to improve antioxidant enzyme activities as well as adenosine triphosphate (ATP) generation while subjected to acetic acid stress (Zhang, *et al*., 2015). It has been proposed that *SET5* may play a role in the epigenetic control of genes that regulate stress responses involved in heterologous protein production, as these strains showed improved activity in the presence of tunicamycin, which is known to trigger unfolded protein stress.

The *PDI1* gene plays a critical role in disulphide bond formation, resolving non-native disulphide bonds, and assisting with protein folding in the ER and isomerization reactions (den Haan, *et al*., 2013). This disulphide bond formation is necessary for proper protein folding, and the formation of incorrect disulphide bonds has been implicated in the accumulation of ROS. Overexpression of *PDI1* was capable of significantly increasing the secretion of all three cellulosic enzymes (Song, *et al*., 2018) and has also led to improved secretion of other heterologous proteins (den Haan, *et al*., 2013).

1.11.4. SNARE proteins

In eukaryotes, membrane fusion events along the endocytic and secretory pathways often, if not always, involve SNARE proteins (van Zyl, *et al*., 2014). SNAREs are a class of type II membrane protein in which the C-terminal segment serves as a membrane anchor while a short α-helical motif, which is roughly 70 amino acids in length, distinguishes different SNAREs from each other (van Zyl, *et al*., 2016). SNARE proteins have been identified on the ER, the Golgi membrane, the vacuole/lysosome, the plasma membrane, and the vesicles derived from these respective membranes. They facilitate protein trafficking between the various membrane-enclosed organelles and the plasma membrane during secretion. While taking part in these events, their role also involves ensuring specificity and stability of these events (van Zyl, *et al*., 2016).

SNARE proteins are divided into two classes, dependent on the localization of their fusion activity (van Zyl, *et al*., 2016). Vesicle (v)-SNAREs are those present on protein transport vesicles whilst their related partner proteins, target (t)-SNAREs are located on the particular target membranes that the corresponding v-SNARE will bind to. The v- and t-SNARES are capable of recognising one another and form trans-SNAREs or a SNARE-pin in which the SNAREs form complexes that facilitate SNARE-mediated exocytosis (van Zyl, *et al*., 2014), bringing the respective lipid bilayers into proximity, forming an energetically favourable state for fusion (den Haan, *et al*., 2013).

The overexpression of SNARE encoding genes associated with vesicle fusion events, especially those promoting the Golgi to exocytosis phase, has been applied in numerous studies as a method of increasing heterologous protein expression (Idris, *et al*., 2010). SNARE proteins SED5, SEC22, BOS1 and BET1 play the role of facilitating the targeting and fusion of anterograde vesicles to the *cis*-Golgi in the ER-to-Golgi subsection of the yeast secretion pathway (Figure 1.10). The functional t-SNARE sub-complex of the yeast ER-to-Golgi SNARE complex consists of BOS1, SEC22 and SED5 and small quantities of the v-SNARE BET1. The t-SNARE SED5 binds with BOS1 and SEC22, to form a functional t-SNARE ternary complex that can receive transport vesicles from the ER, harbouring the v-SNARE BET1 (van Zyl, *et al*., 2016). In a study by van Zyl and co-workers (2016) overexpression of *BET1*, *SEC22* and *SED5* led to the between 22% and 68% increases in the levels of secreted *T. emersonii* CBH activity. They did however note that only the *SED5* was able to enhance heterologous *S. fibuligera* BGL secretion levels.

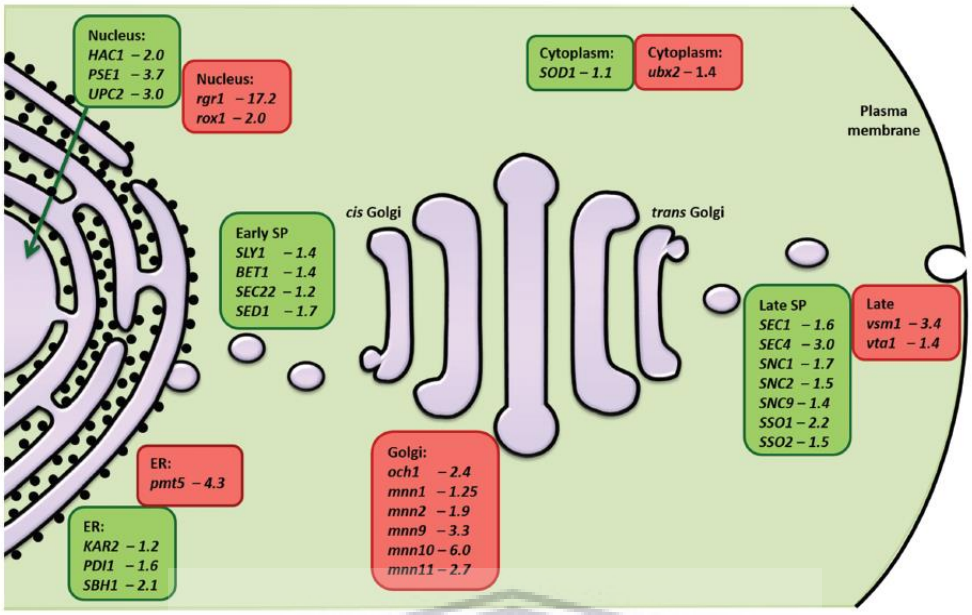

Figure 1.10: Illustration of the various engineering attempts that have been made on the yeast secretory pathway; with green boxes listing gene overexpression and red boxes genes that were deleted *(Kroukamp, et al., 2018).*

Van Zyl and collaborators (2014) overexpressed the *SNC1*, *SNC9* and *SSO1* SNARE encoding genes (see Figure 1.10) and effectively increased the secretion of BGL between 20% and 40%. In comparison, the CBH activity was improved by the overexpression of *SNC1*, *SNC2*, *SNC9*, *SSO1* and *SSO2* by between 23% and 74% (Kroukamp, *et al.*, 2018). It was initially thought that the *SSO1* t-SNARE and its paralog *SSO2* were functionally redundant, however, they have since been differentiated with *SSO1* having been shown to play a central role in sporulation (van Zyl, *et al.*, 2014). Both *SSO1* and *SSO2*, have been reported to increase protein secretion 4- to 6-fold when overexpressed in *S. cerevisiae* using multicopy plasmids (den Haan, *et al.*, 2013). The v-SNARE paralogs *SNC1* and *SNC2* were also reported to be functionally redundant (van Zyl, *et al.*, 2014). When both *SED5* and *SSO1* were simultaneously overexpressed, the resulting strain showed an improvement in *Sf*-Cel3A production of approximately 130% which surpassed the titres obtained when these proteins were overexpressed individually (22% for *SED5* and 49 % for *SSO1*).

Table 1.2: SNARE proteins shown to have a positive effect on the secretion of cellulases when overexpressed in recombinant cellulolytic yeast strains.

PROTEIN	RELEVANT NATIVE FUNCTION	GENE SIZE	IMPROVED SECRETION OF:
BET1 (BLOCKED EARLY IN TRANSPORT)	Type II membrane protein required for vesicular transport between the endoplasmic reticulum and Golgi complex (Newman, *et al*., 1990)	560bp	*Saccharomycopsis fibuligera* BGL *Talaromyces emersonii* CBH (van Zyl, *et al*., 2016)
PSE1 (PROTEIN SECRETION ENHANCER)	Karyopherin/importin that interacts with the nuclear pore complex; acts as the nuclear import receptor for specific proteins (Chow, *et al*., 1992)	3270bp	*S. fibuligera* BGL *Trichoderma reesei* EG1 *Neocallimastix patriciarum* CBH2 (Kroukamp, *et al*., 2013)
SEC9 (SECRETORY)	t-SNARE protein required for secretory vesicle-plasma membrane fusion; bringing membranes into close proximity for energetically favourable fusion (Kroukamp, *et al*., 2018)	1956bp	*T. emersonii* CBH *S. fibuligera* BGL (van Zyl, *et al*., 2014)
SED5 (SUPPRESSOR OF ERD2 DELETION)	*cis*-Golgi t-SNARE syntaxin; required for vesicular transport between the ER and the Golgi complex; binds at least 9 SNARE proteins (Peng, *et al*., 1999)	1023bp	*T. emersonii* CBH *S. fibuligera* BGL (van Zyl, *et al*., 2016)
SEC18 (SECRETORY)	AAA ATPase and SNARE disassembly chaperone; required for vesicular transport between ER and Golgi, the 'priming' step in homotypic vacuole fusion, autophagy, and protein secretion; releases SEC17 from SNAP complexes (Steel, *et al*., 1999)	2277bp	*S. fibuligera* BGL *T. reesei* EG2 (D. Vogel, honours thesis, UWC, 2015)
SNC1 (SUPPRESSOR OF THE NULL ALLELE OF CAP)	Vesicle membrane receptor protein (v-SNARE); involved in membrane fusion between Golgi-derived secretory vesicles with the plasma membrane bringing membranes into close proximity for energetically favourable fusion; proposed to be involved in endocytosis (van Zyl, *et al*., 2014)	467bp	*T. emersonii* CBH *S. fibuligera* BGL (van Zyl, *et al*., 2014)
SSO1 (SUPRESSOR OF SEC ONE)	Plasma membrane t-SNARE; involved in fusion of secretory vesicles at the plasma membrane and in vesicle fusion during sporulation; forms a complex with SEC9 that binds v-SNARE SNC2; syntaxin homolog (Nakanishi, *et al*., 2006)	873bp	*T. emersonii* CBH *S. fibuligera* BGL (Xu, *et al*., 2013; van Zyl, *et al*., 2014; van Zyl, *et al*., 2016)

The *PSE1* protein is a member of the β-karyopherin family and has been implicated in the export of messenger ribonucleic acid (mRNA) out of the nucleus to the cytosol (see Table 1.2) (Isoyama, *et al*., 2001). The overexpression of this protein is purported to increase the general secretion of cellulolytic proteins (Kroukamp, *et al*., 2013). Due to its proposed role, it was postulated that the

increase in secreted protein quantities is likely due to changes at the transcription/translation steps in the protein secretion pathway. In Table 1.2 more details of the above-mentioned SNARE proteins shown to have positive effects on heterologous cellulase secretion, which will be used in this study, are listed with their respective functions.

1.12. Objectives of the Study

Past research suggests that recombinant cellulases produced by candidate CBP yeasts would need to work in a synergistic manner similar to fungal cellulases, whereby two or more cellulases are present in a specific ratio and are essential components of the full hydrolysing activity (den Haan, *et al*., 2013; Liu, *et al.*, 2017; Yamada, *et al.*, 2010). One route to enzyme cost reduction in biofuel production is thus identifying key essential enzymes and adapting their ratios in heterologous expression (Davison, *et al*., 2020). To date, only a handful of studies have demonstrated the capability of recombinant *S. cerevisiae* to ferment cellulosic substrates to ethanol without the addition of exogenous enzymes (den Haan, *et al*., 2007; Inokuma, *et al*., 2017; Davison, *et al*., 2019). One such study was the engineering of a strain, through cocktail δ-integration, to produce the core set of heterologous cellulases in a ratio optimised manner for hydrolysis of crystalline cellulose (Liu *et al.* 2017). However, these cell-surface engineered strains were not yet feasible for commercialization due to the low bioconversion efficiency of LCB (Chen, *et al*., 2018). In several other studies, it was shown that heterologous cellulase secretion in yeast could be significantly enhanced through rational strain engineering, including the overexpression of SNARE encoding genes (Van Zyl *et al.*, 2014; Van Zyl *et al*., 2016; Kroukamp *et al*., 2018). In this work, we carried out a comprehensive study to enhance cellulase activity of strains previously engineered for CBP by Liu and co-workers (2017). This was achieved by testing the effects of gene targets previously reported to improve secreted levels of BGL, EG and CBH on the secretion of surface tethered cellulases. Gene targets most likely to improve BGL, EG and CBH secretion were selected from literature: *PSE1* and various SNARE genes namely *SEC18, SSO1, SNC1*, *SEC9*, *SED5*, and *BET1* were thus identified (Table 1.2). To test the effect of these genes on the engineered cellulolytic strains the following objectives were set:

- Delta integration plasmids (pBKD/BZD/BHD) carrying the secretion enhancement genes were introduced into EG, BGL and CBH bearing *S. cerevisiae* strains obtained from our Japanese collaborators (Prof. Tomohisa Hasunuma and Prof. Kentaro Inokuma, Kobe University, Japan).
- The individual cellulase activities and overall cellulose conversion levels were assayed.
- Changes in strain physiology: growth, ethanol tolerance, osmotic tolerance, tunicamycin resistance and other fermentation stressors were also tested

- Fermentation of microcrystalline cellulose (avicel) to ethanol by selected strains was tested and high pressure liquid chromatography was performed to identify ethanol production and glucose utilization.

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

2. Chapter 2: Materials and Methodology

2.1. Media and Culturing Conditions

Media components and chemicals used were all laboratory grade standard and purchased from Merck or Sigma unless otherwise stated. All yeast strains used in this study were derived from strain *S. cerevisiae* BY4741 (MATa *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0). Strains were stored at −80°C in growth medium supplemented with 15% (v/v) glycerol. The *S. cerevisiae* strains were routinely cultivated in yeast peptone dextrose (YPD) (10g/L yeast extract, 20g/L peptone, 20g/L glucose) medium on a rotary shaker (180rpm) at 30°C. For the generation of yeast transformants, all *S. cerevisiae* transformants were selected on YPD agar (20g/L agar) supplemented with 200µg/mL geneticin (G418) disulphate (Invitrogen) or 200µg/mL hygromycin (Invitrogen) as required. Bacterial cells were grown in Luria-Bertani (LB) broth (10g/L tryptone, 5g/L yeast extract, and 10g/L sodium chloride) containing 100µg/L ampicillin at 37°C on a rotary mixer.

2.2. Heterologous Strain Construction

Descriptions of the yeast strains and plasmids that were used in this study are summarized in Table 2.1 and Table 2.2. The laboratory strain S288c (ATCC 204508) was included in this study to be used as reference strain. Several yeast strains used in this study were provided by Prof. Tomohisa Hasunuma (Kobe University, Japan). These strains were described by Liu and collaborators (2017) and Inokuma and co-workers (2014). Three codon-optimized genes encoding *Trichoderma reesei* EGII, *Talaromyces emersonii* CBHI, and *Chrysosporium lucknowense* CBHII were expressed and used for assembly of enzyme cocktails in a BGL-displaying *S. cerevisiae* BY4741. Strains were constructed using the promoter and the GPI anchoring region derived from *S. cerevisiae SED1*. The secretion signal peptide of BGLI was derived from *Rhizopus oryzae* glucoamylase, while EGII, CBHI and CBHII were produced with their native secretion signals. (Liu, *et al.*, 2017). The plasmids containing the genes encoding the relevant SNARE proteins used for transformation were isolated from the relevant *Escherichia coli* strains using the cetyltrimethylammonium bromide (CTAB) method (Sambrook, 2006). The geneticin disulphate resistance gene (G418) was used as the selectable marker for the first round of transformations while the zeocin resistance gene (Sh *ble*) was used as the selectable marker gene for the double expression strains. Plasmids were

linearized with Bst1107I (ThermoFisher Scientific) after which transformation of the yeast strains (described below, Table 1) was carried out using the LiOAc/DMSO protocol (Hill, *et al*., 1991). An overnight expression step was conducted in YPD media containing 200µg/mL of the appropriate antibiotic at 30°C. Up to forty colonies from each transformant strain were selected for screening to account for clonal variance and transformants with the highest cell-specific activity were chosen for subsequent assays which were performed in triplicate.

Table 2.1: Description of yeast strains used in this study.

YEAST STRAIN	ABBREVIATION	RELEVANT FEATURES	SOURCE
***S. cerevisiae* 288C**	S288C	Haploid *S. cerevisiae* lab strain (Reference stain)	ATCC 204508
***S. cerevisiae* BY-BG-SS**	BYBGSS	*S. cerevisiae* BY4741 with single integration of *A. aculeatus* BGL gene in BY4741 expressed on the cell surface.	(Inokuma, *et al*., 2014)
***S. cerevisiae* EG-D-CBH1-D-CBH2-D**	ECC	*S. cerevisiae* BY4741 with single integration of BGL, EG, CBHI, and CBHII	(Liu, *et al.*, 2016)
***S. cerevisiae* δ-integrated A26 strain**	δA26	*S. cerevisiae* BY4741 with ratio optimized BGL, EG, CBHI, and CBHII genes through δ-integration.	(Liu, *et al.*, 2017)
***S. cerevisiae* BYBGSS + pBKD1-BET1**	BYBGSS_BET1	*S. cerevisiae* BYBGBS strain transformed with pBKD1-BET1	This study
***S. cerevisiae* BYBGSS + pBKD1-PSE1**	BYBGSS_PSE1	*S. cerevisiae* BYBGBS strain transformed with pBKD1-PSE1	This study
***S. cerevisiae* BYBGSS + pBKD1-SEC9**	BYBGSS_SEC9	*S. cerevisiae* BYBGBS strain transformed with pBKD1-SEC9	This study
***S. cerevisiae* BYBGSS + pBKD1-SEC18**	BYBGSS_SEC18	*S. cerevisiae* BYBGBS strain transformed with pBKD1-SEC18	This study
***S. cerevisiae* BYBGSS + pBKD1-SED5**	BYBGSS_SED5	*S. cerevisiae* BYBGBS strain transformed with pBKD1-SED5	This study
***S. cerevisiae* BYBGSS + pBKD1-SNC1**	BYBGSS_SNC1	*S. cerevisiae* BYBGBS strain transformed with pBKD1-SNC1	This study
***S. cerevisiae* BYBGSS + pBKD1-SSO1**	BYBGSS_SSO1	*S. cerevisiae* BYBGBS strain transformed with pBKD1-SSO1	This study
***S. cerevisiae* EG-D-CBH1-D-CBH2-D + pBKD1-BET1**	ECC_BET1	*S. cerevisiae* EG-D-CBH1-D-CBH2-D strain transformed with pBKD1-BET1	This study

***S. cerevisiae* EG-D-CBH1-D-CBH2-D + pBKD1-PSE1**	ECC_PSE1	*S. cerevisiae* EG-D-CBH1-D-CBH2-D strain transformed with pBKD1-PSE1	This study
***S. cerevisiae* EG-D-CBH1-D-CBH2-D + pBKD1-SEC9**	ECC_SEC9	*S. cerevisiae* EG-D-CBH1-D-CBH2-D strain transformed with pBKD1-SEC9	This study
***S. cerevisiae* EG-D-CBH1-D-CBH2-D + pBKD1-SEC18**	ECC_SEC18	*S. cerevisiae* EG-D-CBH1-D-CBH2-D strain transformed with pBKD1-SEC18	This study
***S. cerevisiae* EG-D-CBH1-D-CBH2-D + pBKD1-SED5**	ECC_SED5	*S. cerevisiae* EG-D-CBH1-D-CBH2-D strain transformed with pBKD1-SED5	This study
***S. cerevisiae* EG-D-CBH1-D-CBH2-D + pBKD1-SNC1**	ECC_SNC1	*S. cerevisiae* EG-D-CBH1-D-CBH2-D strain transformed with pBKD1-SNC1	This study
***S. cerevisiae* EG-D-CBH1-D-CBH2-D + pBKD1-SSO1**	ECC_SSO1	*S. cerevisiae* EG-D-CBH1-D-CBH2-D strain transformed with pBKD1-SSO1	This study
***S. cerevisiae* δ-integrated A26 strain + pBKD1-BET1**	δA26_BET1	*S. cerevisiae* δ-integrated A26 strain transformed with pBKD1-BET1	This study
***S. cerevisiae* δ-integrated A26 strain + pBKD1-PSE1**	δA26_PSE1	*S. cerevisiae* δ-integrated A26 strain transformed with pBKD1-PSE1	This study
***S. cerevisiae* δ-integrated A26 strain + pBKD1-SEC9**	δA26_SEC9	*S. cerevisiae* δ-integrated A26 strain transformed with pBKD1-SEC9	This study
***S. cerevisiae* δ-integrated A26 strain + pBKD1-SEC18**	δA26_SEC18	*S. cerevisiae* δ-integrated A26 strain transformed with pBKD1-SEC18	This study
***S. cerevisiae* δ-integrated A26 strain + pBKD1-SED5**	δA26_SED5	*S. cerevisiae* δ-integrated A26 strain transformed with pBKD1-SED5	This study
***S. cerevisiae* δ-integrated A26 strain + pBKD1-SNC1**	δA26_SNC1	*S. cerevisiae* δ-integrated A26 strain transformed with pBKD1-SNC1	This study
***S. cerevisiae* δ-integrated A26 strain + pBKD1-SSO1**	δA26_SSO1	*S. cerevisiae* δ-integrated A26 strain transformed with pBKD1-SSO1	This study
***S. cerevisiae* EG-D-CBH1-D-CBH2-D + pBKD1-SEC9 + pBZD1-SED5**	ECC_SEC9_SED5	*S. cerevisiae* EG-D-CBH1-D-CBH2-D strain transformed with pBKD1-SEC9 and pBZD1-SED5	This study
***S. cerevisiae* EG-D-CBH1-D-CBH2-D + pBKD1-PSE1 + pBZD1-SED5**	ECC_PSE1_SED5	*S. cerevisiae* EG-D-CBH1-D-CBH2-D strain transformed with pBKD1-PSE1 and pBZD1-SED5	This study

***S. cerevisiae* δ-integrated A26 strain + pBKD1-SSO1 + pBZD1-SED5**	δA26_SSO1_SED5	*S. cerevisiae* δ-integrated A26 strain transformed with pBKD1-SSO1 and pBZD1-SED5	This study
***S. cerevisiae* δ-integrated A26 strain + pBKD1-SNC1 + pBZD1-SED5**	δA26_SNC1_SED5	*S. cerevisiae* δ-integrated A26 strain transformed with pBKD1-SNC1 and pBZD1-SED5	This study

Table 2.2: Description of plasmids used in this study.

PLASMID	GENOTYPE	GENE SIZE	SOURCE
pBKD1-PSE1	*PGK_P-PSE1-PGK_T* (G418 selection)	3270bp	(Kroukamp, *et al*., 2013)
pBKD1-SEC18	*PGK_P-SEC18-PGK_T* (G418 selection)	2277bp	(D. Vogel, UWC Honours thesis, 2015)
pBKD1-SED5	*PGK_P-SED5-PGK_T* (G418 selection)	1023bp	(van Zyl, *et al*., 2016)
pBKD1-SSO1	*PGK_P-SSO1-PGK_T* (G418 selection)	873bp	(Van Zyl, *et al*., 2014)
pBKD1-SNC1	*PGK_P-SNC1-PGK_T* (G418 selection)	467bp	(Van Zyl, *et al*., 2014)
pBKD1-SEC9	*PGK_P-SEC9-PGK_T* (G418 selection)	1956bp	(Van Zyl, *et al*., 2014)
pBKD1-BET1	*PGK_P-BET1-PGK_T* (G418 selection)	560bp	(van Zyl, *et al*., 2016)
pBZD1-SED5	*PGK_P-SED5-PGK_T* (Zeocin selection)	1023bp	(van Zyl, *et al*., 2016)

2.3. PCR Confirmation

Transformation of the plasmids containing the relevant genes encoding the SNARE proteins was confirmed via PCR using the PGK-L and PGK-R primers listed in Table 3. These primers were designed to amplify any the region spanning the *S. cerevisiae PGK1* promoter to the terminator. This would include the native *PGK1* open reading frame and the relevant gene introduced into transformants (Table 2). A single colony from each transformation reaction was picked for analysis by PCR. PCR amplification with SYBR Green PCR Master Mix (ThermoFisher Scientific) as recommended by the manufacturer was performed using a MiniAmp Plus Thermal Cycler (ThermoFisher Scientific). PCR products were then separated on a 1 % (w/v) agarose gel. Representative gels are shown in Appendix A4-6. All transformations were confirmed in this way.

Table 2.3: Primers used for PCR amplification of transformants generated in this study.

PRIMER NAME	OLIGONUCLEOTIDE SEQUENCE (5' – 3')
PGK-L	CTAATTCGTAGTTTTTCAAGTTCTTAGATGC
PGK-R	ACTATTATTTTAGCGTAAAGGATGGGG

2.4. Enzyme Assays

Transformants and each parental strain, used as the reference strain, were initially screened after cultivation in 5 mL YPD at 30°C for 48 hours on an orbital shaker at 180rpm. Transformants with the highest normalized BGL, EG and CBH activity compared to the reference strains were subsequently assayed in biological triplicates. When selecting transformants, improved CBH activity took preference over EG and BGL activity. Yeast strains were inoculated in triplicate in 100 mL Erlenmeyer flasks containing 10mL YPD and cultivated at 30˚C for 72 hours, on an orbital shaker at 180rpm. All assays were performed on the total culture and supernatant, and the cells were removed by centrifugation from the total culture samples prior to further analysis.

To evaluate the β-glucosidase (BGLI) activity of the recombinant strains, enzyme assays were performed in triplicate at 24 hours, 48 hours, and 72 hours after inoculation of the selected heterologous transformants, over-expressing different combinations of the SNARE genes. Assays to determine BGLI activity were carried out using *p*-nitrophenyl-β-D-glucopyranoside (pNPG; Sigma) as the substrate, at 50°C with reaction times of 2 minutes as was previously described (Van Zyl, *et al*., 2014). The absorbance of 100µL of the supernatant of the assay mixture was measured at 400nm using a FLUOstar Omega Microplate Reader (BMG LABTECH) and compared with a standard curve set between 0.075 and 1.25mM pNP (see Appendix, Figure A1) to the determine how much of the substrate was cleaved.

The extracellular CBH activity of transformants was also evaluated at 24 hours, 48 hours, and 72 hours using *p*-nitrophenyl-β-D-cellobioside (pNPC; Sigma) as a substrate, with reactions carried out for 10 minutes at 50°C as was previously described (Van Zyl, *et al*., 2014). CBH production was also measured by activity on the soluble fluorescent substrate 4-methylumbelliferyl-β-D-lactoside (MULac; Sigma) using the method previously described by Ilmén and colleagues (2011) with a reaction time of 30 minutes at 37°C and compared to a (methylumbelliferone) MU standard curve set between 0.63µM and 20µM (Appendix, Figure A2).

To evaluate the EG activity from transformants, a carboxymethylcellulose / dinitro-salicylic acid (CMC/DNS) assay was used and incubated for 10 minutes at 50°C as described by Den Haan and co-workers (2007). The DNS glucose standard curve ranged between 0.5–1.5mM (see Appendix, Figure A3) with OD values read at 540nm. Dry cell weight (DCW) was estimated from OD_{600} readings and used to normalize the volumetric activity values of the yeast cultures. Enzyme

activities were expressed as units/mg or units/g DCW, where one unit was defined as the amount of enzyme required to release 1µmol of reducing sugar or equivalent per minute. Assays were performed in biological and technical triplicates and values are given as averages of these repeats with standard deviation indicated. Significant differences between activities attained were investigated using a two-tailed T-test, assuming unequal variance. A p-value lower than 0.05 was deemed significant.

2.5. Cellulose Conversion

Strains to be tested were inoculated into YPD media and grown at 30 ˚C for 48 hours. A substrate mixture containing 2% (w/v) avicel, 500mM sodium acetate (pH 5.0) and 0.02% (w/v) sodium azide was made up and continuously stirred to ensure homogeneity and that the avicel would not settle to the bottom. 100mM (v/v) methylglyoxal was added to the substrate mix. The methylglyoxal prevented the assimilation of glucose released during the assay by yeast cells so that the level of glucose released could be measured. In a 96-deep-well plate a 1:1 mixture of the substrate mix and yeast culture were added to a final volume of 600µL, performed in triplicate. A sample was taken at 0 hours as a baseline measurement for background sugar. The reaction was performed at 35°C, shaking at 1000rpm in a Heidolph Titramax 1000 microplate shaker/incubator. The mixture was assayed for cellulose conversion by measuring the amount of glucose released. This cellulolytic activity represents the degradation of the substrate via all the cellulase enzymes present in the reaction broth. Samples were taken at 48 hours and 72 hours. The amount of glucose in the supernatant was determined by performing the dinitrosalicylic acid (DNS) assay method after centrifuging 200µL samples and carefully transferring the supernatant to a clean 96 well PCR plate. Absorbance was determined at an OD of 540nm. Activity for cellulose conversion was calculated as the amount of substrate (avicel) hydrolysed, by determining the amount of glucose released during the assay.

2.6. Growth Analysis

Growth analysis was performed by creating a seed culture in test tubes by inoculating isolates into 5mL YPD media which was incubated on a rotary shaker at 180rpm overnight at 30°C. The *S. cerevisiae* S288c strain was included as a reference. The resulting pre-cultures were normalized to OD_{600} = 0.1 into 100mL Erlenmeyer flasks containing 20mL YPD media. Flasks were incubated

on a rotary shaker at an agitation speed of 180rpm at 30°C for the duration of the analysis. Samples were taken hourly for the first 16 hours. Samples were taken every 3 hours thereafter until 48 hours when isolates reached stationary phase. Samples were appropriately diluted and OD_{600} readings were taken using FLUOstar Omega Microplate Reader (BMG LABTECH) as an indicator of cell growth. Growth analysis was performed with biological triplicates for each strain and values are given as averages of these repeats with standard deviation indicated.

2.7. Evaluation of Fermentation Stressors

To screen for innate tolerance capabilities between the parental (in this case S288c, BY-BG-BS, EG-d-CBHI-d-CBH2 and δ-integrated A26), and transformed strains, the yeast strains were inoculated into 100mL conical flasks containing YPD media and grown at 30˚C for 48 hours, agitated at 180rpm on a rotary shaker. Absorbance at OD_{600} was taken and all cultures subsequently standardized to the same OD with sterile YPD media. Tenfold serial dilutions of these were then spotted onto YPD agar plates supplemented with the appropriate inhibitors as detailed below or incubated at different temperatures. Cells were grown for 30°C for 48 hours unless otherwise stated.

To evaluate the strains' resistance to ER stress, the antibiotic tunicamycin (of 0.5µg/mL and 0.8µg/mL), which inhibits glycosylation was supplemented into the media. To test the ability to grow after final product formation, media was supplemented with ethanol (10%, 20% and 30%). For evaluation of osmotic stress tolerance media was supplemented with NaCl (1M and 1.2M). Isolates were spot inoculated on YPD agar and also incubated at 30˚C and 35˚C to observe any changes under heat stress.

2.8. Fermentation of Crystalline Cellulose

Strains were inoculated from fresh plates into 10mL YPD and incubated on an orbital shaker overnight at 30˚C, at an agitation of 180rpm. The 10mL seed cultures were then inoculated into a 1000mL Erlenmeyer flask containing 400mL YPD broth and incubated with shaking at 30˚C for 48 hours. Cultures were then transferred to sterile centrifuge bottles and centrifuged at 1000x*g* for 10 minutes. The supernatant was removed, and 30mL sterile water added, and the pellet resuspended. The cell suspension was then transferred to a 50 mL Greiner tube. The suspension was centrifuged at 1000x*g* for 10 minutes and the water removed. A second wash with 30mL water

was performed and wet cell weight was recorded. Using these recorded weights, cells were normalized to 600g wet cell weight/L. Fermentation media was set up in 30mL rubber stoppered serum bottles containing sterile 5X yeast peptone (YP) media (20g/L yeast extract, 40g/L peptone), 10g/L avicel, and sterile water. Cells were added to the reaction mixture to a final concentration of 150g wet cell/L together with ampicillin (100µg/ml) and streptomycin (100µg/ml) to suppress bacterial growth. The serum bottles were sealed with rubber stoppers to maintain the oxygen-limited conditions. A 0.8 × 25mm syringe needle, plugged with cotton wool was pierced into the rubber stoppers to function as CO_2 outlets. Fermentation reactors were incubated with shaking at 180rpm, for 96 hours at 30˚C. Samples to the volume of 1200µL were taken at 0, 24, 48, 72 and 96 hours and frozen at -20°C prior to high-performance liquid chromatography analysis.

2.9. High-performance liquid chromatography (HPLC)

Fermentation samples were centrifuged at 13000x*g* for 10 minutes and the supernatant transferred to a new Eppendorf tube, making sure to avoid the pellet. A 10% (v/v) sulphuric acid (H_2SO_4) solution was added to each sample and vortexed briefly. Samples were filtered using a 0.22µm filter into HPLC tubes. Ethanol, glycerol, acetic acid, and glucose concentrations were quantified by HPLC equipped with a BioRad guard (part # 125-0129) and refractive index (RI) detector. The compounds were separated on a BioRad Aminex HPX-87H (part #125-0140) 7.8 x 300mm column at a temperature of 65°C with 5mM H_2SO_4 as mobile phase at a flow rate of 0.7mL/min and detected using an RI detector.

3. Chapter 3: Results and Discussion

3.1. Enzyme Activity

To address the problem of native *S. cerevisiae* lacking the necessary cellulolytic enzymes needed for hydrolysation of cellulose, heterologous expression of cellulolytic enzymes, more specifically BGL, EG and CBH have been pursued for many years (Inokuma, *et al*., 2014). As mentioned in Chapter 1, Strains used for the purpose of whole-cell biocatalysis for simultaneous saccharification and fermentation have also been constructed in which cellulolytic enzymes are tethered to the cell surface by means of a GPI-anchoring system. Here we attempted to improve the cellulolytic ability of previously constructed strains that had cellulolytic enzymes displayed on the cell surface (Liu, *et al*., 2017). In this study the BY-BG-SS, EG-d-CBH1-d-CBH2-d and δ-integrated A26 strains were all successfully transformed with plasmids containing the relevant SNARE protein encoding genes (as described in Chapter 2, Table 2.1). The putative BY-BG-SS, EG-d-CBH1-d-CBH2-d and δ-integrated A26 overexpressing transformants were then screened and strains displaying the highest enzyme activity per gram dry cell weight were selected for further study (data not shown). This preliminary screening to isolate transformants displaying the greatest CBH activity among the transformants was carried out on at a minimum of 40 transformants of each strain, to account for the clonal variation inherent to δ-integration of genes (van Zyl, *et al*., 2014). For the EG-d-CBH1-d-CBH2-d and δ-integrated A26 overexpressing transformants, superior CBH activity was favoured over improved EG and BGL activities in these early steps as our focus was on improved breakdown of crystalline cellulose, for which CBH activity is paramount (den Haan, *et al*., 2007). Prior to subsequent assays, all strains were confirmed to contain the relevant secretion enhancement gene via PCR analysis (see Appendix, Figure A4-6).

3.1.1. Strain Expressing BGL Only (Parental Strain: BY-BG-SS)

The BY-BG-SS strain (abbreviated as BYBGSS) is a *S. cerevisiae* BY4741 strain with a single integration of the *Aspergillus aculeatus* BGL gene. This strain was selected since it was reported in a study by Inokuma and collaborators (2014) to have achieved the highest BGL activity with a relatively short cultivation time when compared to other strains. These results were observed in a study which tested different anchoring and promoter regions and was also deemed suitable for hydrolysis of insoluble substrates including lignocellulosic biomass. The single integration of the BGL gene means all resultant transformants derived from this stain were only tested for BGL

activity and not all three of the cellulases of interest. Samples were taken at 48 and 72 hours and pNP-G substrate was used to determine the BGL activity for the resulting transformants of the BYBGSS strains (Figure 3.1). Results were normalised with dry cell weight. The results showed that during the first 48 hours a slight increase of 14% was seen for BYBGSS_SEC9 and a much greater increase of 45% was seen for BYBGSS_BET1. However, after 72 hours of cultivation, there was no discernible increase in BGL activity in any of the transformed strains.

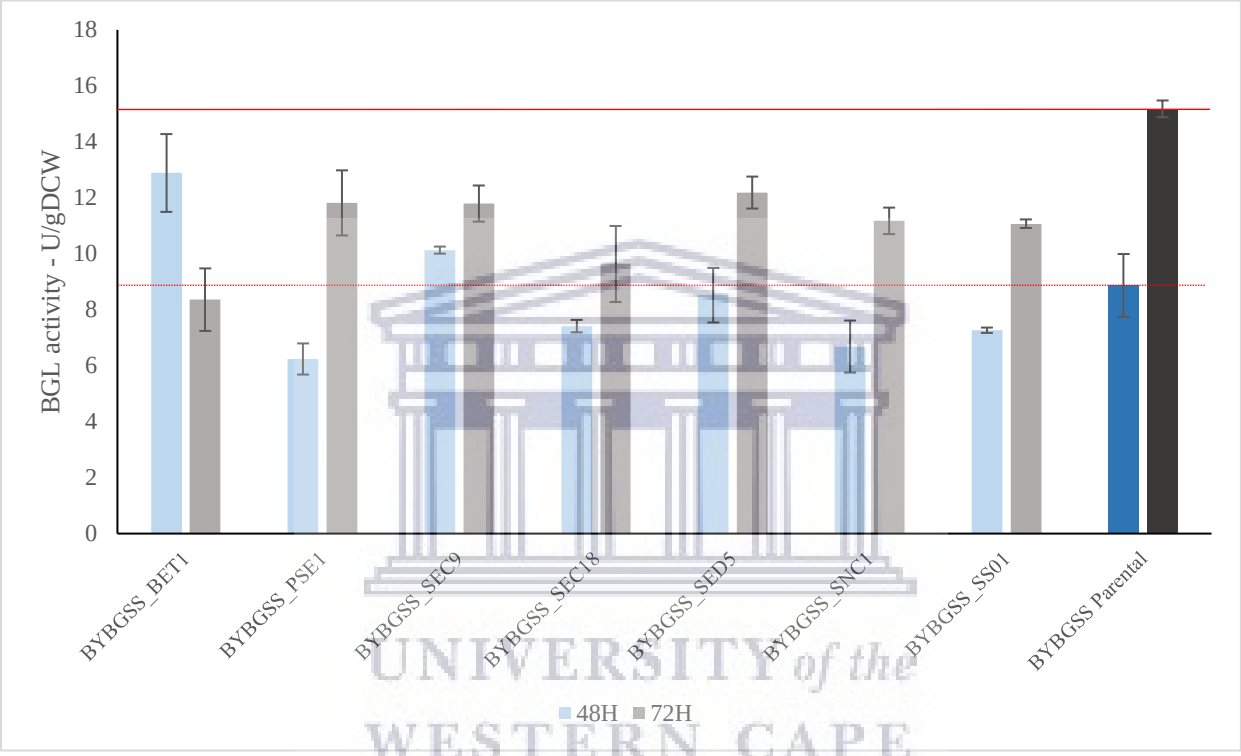

Figure 3.1: The ß-glucosidase (p-nitrophenyl-ß-D-glucopyranoside) activity profiles of the recombinant Saccharomyces cerevisiae BYBGSS strains transformed with one of seven genes reported to improve cellulase secretion. *Values obtained were normalised using dry cell weight (DCW) of each strain and the parental BYBGSS strain was used as reference. Reference activity indicated by red dotted line (24 hours) and solid red line (48 hours). All values represent mean values of assays conducted in triplicate with error bars indicating the standard deviation from the mean value for each strain.*

BET1 has been identified as a type II membrane ER-to-Golgi SNARE protein that facilitates the targeting and fusion of anterograde vesicles to the *cis*-Golgi (van Zyl, *et al*., 2016). When this protein had been expressed in *S. cerevisiae*, it was reported to have yielded a decreased level of BGL enzyme activity according to van Zyl and co-workers (2016). This result is not consistent with the initial 45% increase in BGL activity for the BYBGSS_BET1 transformant reported in this study. The t-SNARE protein, SEC9, was identified to be required for secretory vesicle-plasma membrane fusion during post-Golgi transport, contributing two helical domains to the exocytic

SNARE complex (van Zyl, *et al*., 2014). Van Zyl and colleagues (2014) were able to achieve an increase of 22% in BGL activity when SEC9 was overexpressed in *S. cerevisiae*. The disparity between our results and those published previously, may in part be due to differences in strain background and the BGL encoding gene used, that have been shown to have significant effects on heterologous secretion phenotypes (Gurgu, *et al.*, 2011; Davison, *et al.*, 2016; Davison, *et al*., 2019).

3.1.2. Strain expressing BGL, EG and CBH (Parental Strain: EG-d-CBHI-d-CBHII-d)

EG-d-CBH1-d-CBH2-d (abbreviated as ECC) is a *S. cerevisiae* BY4741 based strain that was constructed through single copy integration of heterologous genes to display BGL, EG, CBHI, and CBHII on the cell surface. Liu and co-workers (2016) noted that this strain displayed significantly higher ethanol production compared to all other strains tested in that study on both amorphous and crystalline cellulose. It also performed at a superior capacity when compared against other cellulolytic strains previously described in other relevant studies. This strain displayed a cell-to-cellulose adhesion and a "tearing" pattern as part of its cellulose-degradation mechanism, which differed from those of strains producing free-form enzymes (Liu, *et al*., 2016).

The BGL activity of the ECC transformant strains generated during this study was tested using pNP-G after 72 hours of cultivation, as seen below in Figure 3.2 (A). The ECC parental strain was used as reference for all subsequent ECC transformants into which the various SNARE proteins had been transformed. After 72 hours, only the ECC_SED5 strain had shown an improvement in BGL activity when compared to that of the parental strain. The EG activity of the ECC transformants was tested on CMC and results are displayed below in Figure 3.2. (B). Following the 72 hour cultivation, most of the strains showed a pronounced increase in the EG activity, and no decrease in activity was detected across any of the strains. Notably, ECC_SED5 displayed the most significant improvement, showing an increase of 231% (p-value = 0.003) compared to the parental strain. The CBH activity for ECC strains, shown in Figure 3.2 (C), were tested using the MU-Lac substrate and it was noted that after 72 hours all strains displayed increased activity when compared to the parental strain. This can be explained by the fact that strains used for further investigation were all selected on the basis of most improved CBH activity during the preliminary screening studies (data not shown).

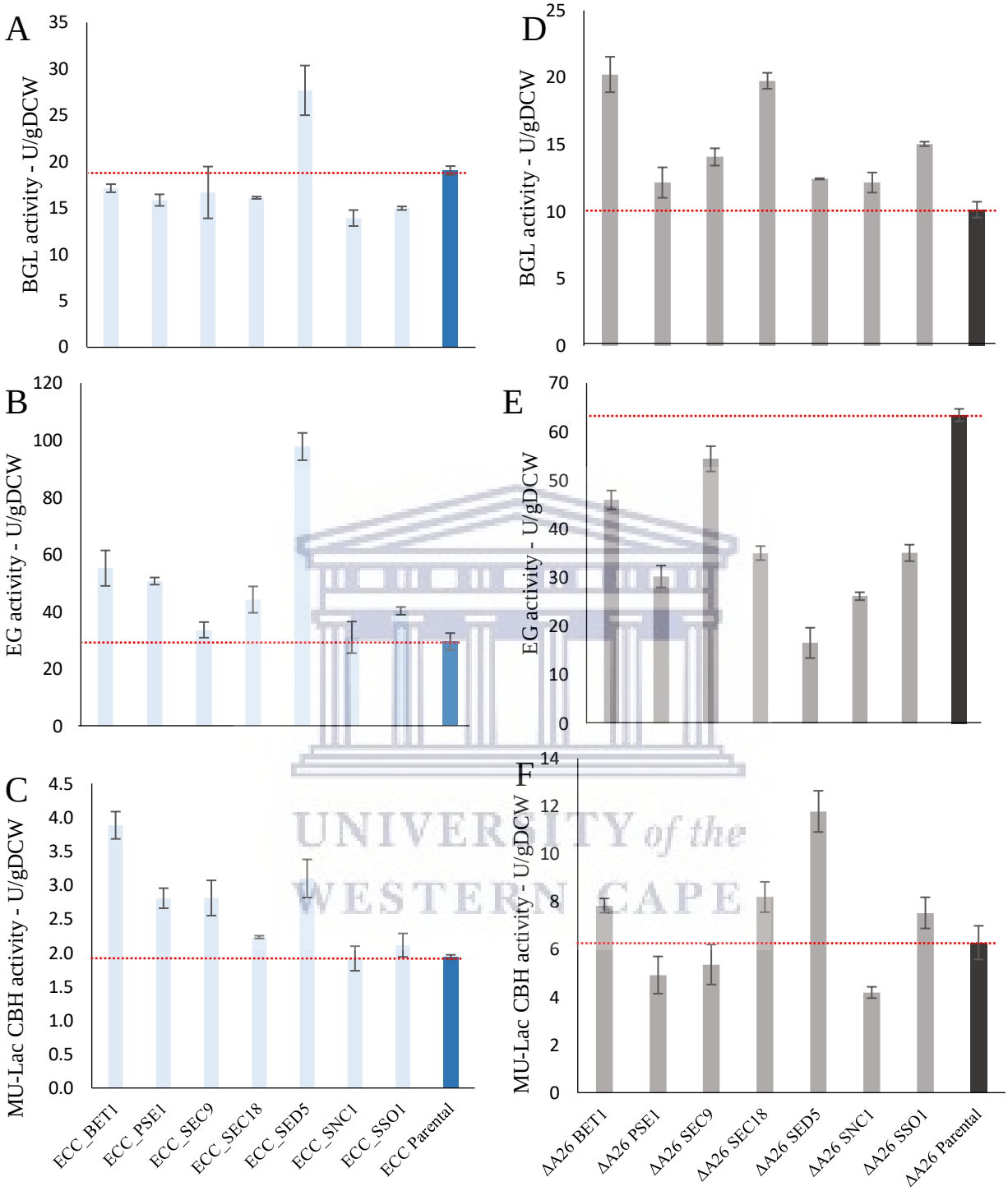

Figure 3.2: Enzyme activity profiles of the recombinant Saccharomyces cerevisiae ECC and ΔA26 strains transformed with one of the seven genes reported to improve cellulase secretion after 72 hours of cultivation. (A&D) β-glucosidase activity on p-nitrophenyl-β-D-glucopyranoside. (B&E) endoglucanase activity on carboxymethyl cellulose. (C&F) cellobiohydrolase activity on 4-methylumbelliferyl-β-D-lactoside. *Values obtained were normalised using dry cell weight of each strain and the parental strains used as reference (activity indicated by red line). All values represent mean values of assays conducted in triplicate with error bars indicating the standard deviation from the mean value for each strain.*

Results of the BGL activity profiles for the ECC transformant strains showed that following a 72 hour cultivation, strain ECC_SED5 outperformed the parental strain producing 27.68U/gDCW of BGL activity. This activity had surpassed that of the parental strain, which had measured 19.02U/gDCW, representing a 46% increase in activity for ECC_SED5. The effects resulting from the overexpression of the SED5 protein had previously been investigated by van Zyl *et al.* (2016) in which they were able to show an improvement in the BGL activity that had surpassed the BGL activities of all other strains tested in the study, similar to the results observed here. Substantial increases in EG activity were seen for all but two strains, indicating that the addition of the various SNARE proteins in this specific *S. cerevisiae* strain (ECC) had not negatively impacted the EG activity and provided significant improvements.

The recorded CBH activity showed improvements of 101, 45, 46, 16, and 61% for ECC_BET1, ECC_PSE1, ECC_SEC9, ECC_SEC18, and ECC_SED5, respectively. The t-SNARE, SSO1, plays a central role in sporulation. Xu and collaborators (2013) reported an increase in CBH activity of 10% when SSO1 was overexpressed in *S. cerevisiae*, corresponding to the results seen in this study, in which an increase of ~9% in CBH activity was observed when measured against the parental strain. ECC_BET1 showed the greatest increase in CBH activity, reaching a 101% increase at 3.88U/gDCW. ECC_SED5 showed a large improvement across all of the enzyme activity testing profiles, further substantiating the claim by van Zyl and colleagues (2016) that the SED5 SNARE protein is a universally effective SNARE target for heterologous protein secretion enhancement. This may illustrate a cellular shortage of this protein, which may be corrected during the overproduction of this specific SNARE protein.

3.1.3. Ratio optimized strain expressing BGL, EG and CBH (Parental Strain: δ-integrated A26)

δ-integrated A26 (abbreviated as ΔA26) is a *S. cerevisiae* BY4741 based strain that was constructed by Liu and co-workers (2017) through a cocktail δ-integration method to display BGL, EG, CBHI, and CBHII enzymes on the cell surface in a ratio optimized manner. With the appropriate ratios of these cellulases, stronger synergies can be achieved (Liu, *et al*., 2017). The study in which this strain was constructed reported the highest ethanol yield from direct conversion of crystalline cellulose by a cellulolytic yeast.

Figure 3.2 (D) displays the results of BGL testing using pNP-G substrate in the strains that had ΔA26 as the background strain. An increase in BGL activity was observed across all the transformant strains. ΔA26_BET1 showed a dramatic increase of 99% when compared to the other strains, with ΔA26_SEC18 also closely resembling this activity profile at an increase of 95%. The ΔA26_SED5 strain showed a small improvement of just 23% despite the overexpression of the same SNARE protein leading to among the greatest increase in enzyme activity for BYBGSS and ECC. Similar to the activity of ΔA26_SED5, ΔA26_PSE1 and ΔA26_SNC1 displayed an increase of only ~20% when compared to the parental strain, indicating the lowest recorded improvement. A similar improvement in the BGL activity was seen for a strain overexpressing the SNC1 protein in a study conducted by Van Zyl and colleagues (2014) in which a 22% increase in the secreted BGL activity was observed. The results of EG activity testing are shown in Figure 3.2. (E). After 72 hours ΔA26_SEC9 displayed only a slight decrease of 14% in EG activity while all other strains experienced a much greater decrease in their activity profiles.

Testing of CBH activity of the ΔA26 based strains on MU-Lac seen in Figure 3.2. (F) illustrated that after 72 hours a decrease in CBH activity was seen in strains ΔA26_PSE1, ΔA26_SEC9 and ΔA26_SNC1, while slight improvements of 25%, 30% and 20% was seen for ΔA26_BET1, ΔA26_SEC18 and ΔA26_SSO1, respectively. A significant increase of 88% (p-value = 0,004) was seen for ΔA26_SED5. This study showed major increases in CBH activity after the addition of this particular SNARE protein to the ΔA26 strain, similar to the trend seen in the ECC_SED5 strain. However, in the ΔA26 background, this SNARE was not capable of increasing the EG activity. The absolute values of CBH activity in the ΔA26 strains were generally far higher than that of the ECC based strains. This is likely due to the way in which the strains were constructed with multiple copies of the CBH encoding gene integrated into the ΔA26 strain compared to the single copy of the ECC strain (Liu, *et al*., 2017). The higher ratio CBH:EG encoding genes, inherent to the ΔA26 strain, may therefore promote the improvement of CBH levels over EG levels.

3.2. Cellulose Conversion

Avicel was used as a means to test the ability of ECC and ΔA26 based transformant strains to hydrolyse crystalline cellulose over a 24 and 48 hour period (Figure 3.3.). As these strains possess the core set of cellulases required to achieve cellulose conversion, it was hypothesized that strains showing greater overall enzyme activity in this assay are likely to have a greater ability to convert the crystalline cellulose into glucose for the eventual production of bioethanol.

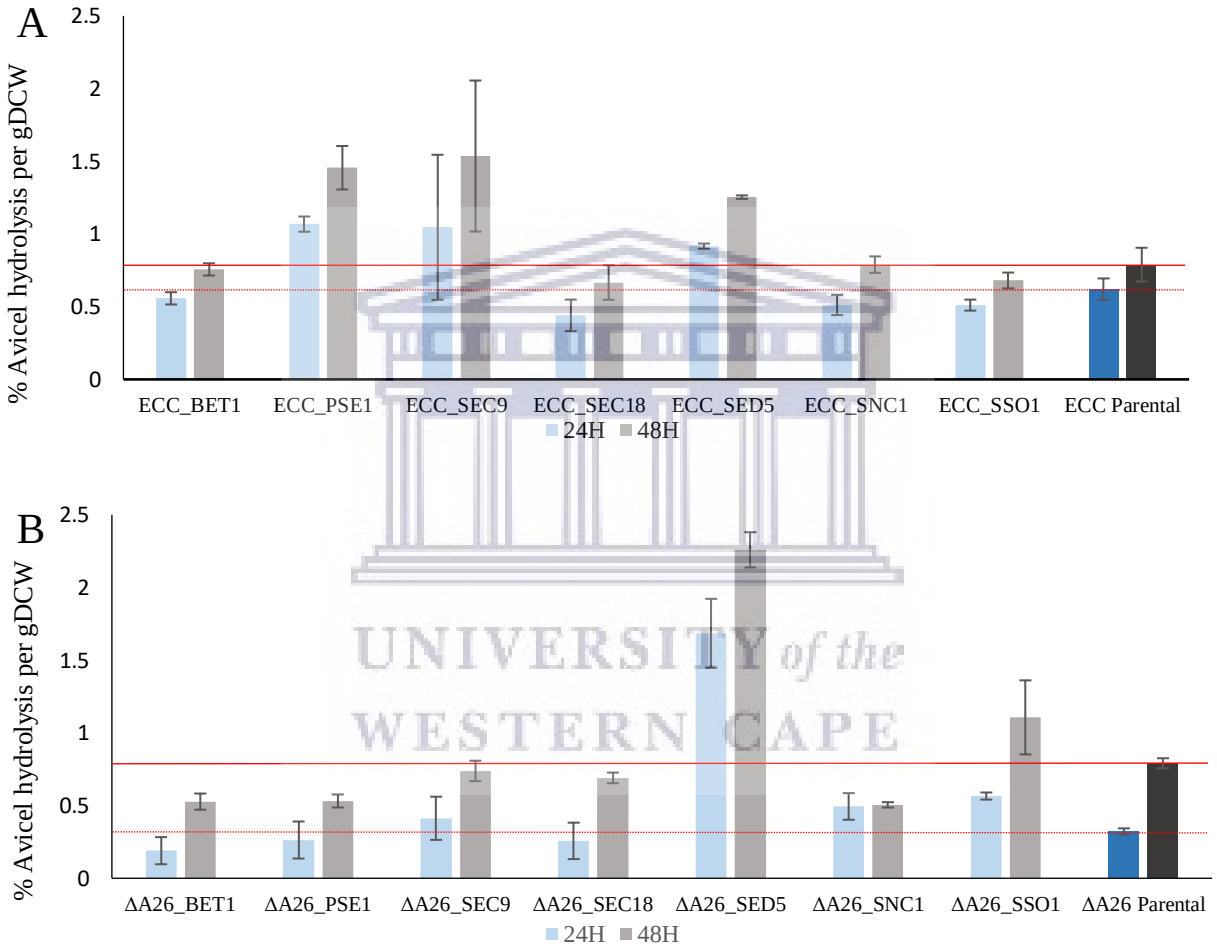

Figure 3.3: The hydrolysis of crystalline avicel by the recombinant Saccharomyces cerevisiae strains after 24 and 48 hours of incubation. (A) ECC transformants (B) ΔA26 transformants. *Values obtained were normalised using dry cell weight (DCW) of each strain and the parental ECC and ΔA26 strain were used as reference. Reference activity indicated by red dotted line (24 hours) and solid red line (48 hours). All values represent mean values of assays conducted in triplicate with error bars indicating the standard deviation from the mean value for each strain.*

After 24 hours, strains ECC_BET1, ECC_SEC18, ECC_SNC1 and ECC_SSO1 had decreased hydrolysis efficiency, when compared to the parental strain as seen in Figure 3.3 (A). A rise in the

hydrolysis efficiency can be seen for ECC_PSE1, ECC_SEC9 and ECC_SED5, where an improvement of 72%, 68% and 48%, respectively was observed. A similar trend was observed after the 48 hours of incubation for all of the strains. The ECC_PSE1 and ECC_SEC9 strains displayed the highest overall hydrolysis efficiency of avicel for both the 24 and 48 hour incubation periods, ECC_SEC9 did however, show large variations between each sample taken and tested for cellulose hydrolysis. These results did not seem to directly correlate with the cellulase activity, as these strains, while having increased enzyme activity in most incidences for all of the cellulases tested, were in fact not the best performing strains. They did however, display improvements to both EG and CBH activities, and this higher titre or improved synergies may be responsible for the improved Avicel hydrolysis observed here.

ΔA26 transformant strains were also tested for hydrolysis efficiency (Figure 3.3. B). After 24 hours, ΔA26_BET1, ΔA26_PSE1 and ΔA26_SEC18 had all displayed a slightly reduced hydrolysis efficiency when compared to that of the parental strain. A notable increase in the hydrolysis efficiency was seen for ΔA26_SEC9 at 28%, ΔA26_SNC1 at 54% and ΔA26_SSO1 at 76% above that of the ΔA26 parental strain. The greatest improvement, however, was observed by the ΔA26_SED5 strain after 24 hours, with a hydrolysis efficiency of 424% above that of the parental strain. It should, however, be noted that all the strains shown to have decreased cellulose conversion efficiencies were in fact among the best BGL performers. In many cases, the overexpression of proteins can cause a number of unforeseen issues such as impaired specific growth rate, delayed fermentation start-up, reduced fermentation rate and/or yield, decreased respiration capacity or reduced biomass yield (Deparis, *et al.*, 2017). This is likely explained by protein production being a costly process for which energy is often expended towards protein translation and ribosomal activity, which is a major limiting factor for the growth rate of the cells. The energy distributed to increasing the BGL load may have reduced the cells' energy expenditure for cellulose conversion. The improved CBH activity observed in ΔA26_SED5 strain (Figure 3.2. C), on the other hand, could explain the increased avicel hydrolysis (Figure 3.3. B) as this cellulase activity is the rate limiting enzyme for conversion of cellulosic biomass (Xu, *et al.*, 2018), thereby showing a direct correlation with the cellulose conversion ability. After 48 hours of incubation, strains ΔA26_BET1, ΔA26_PSE1, ΔA26_SEC9, ΔA26_SEC18, ΔA26_SNC1 showed no improvement in hydrolysis efficiency when compared to the parental strain. Strain ΔA26_SSO1, on the other hand, showed an increase of 40% while ΔA26_SED5 again showed a much greater

improvement of 186%. Both of these strains again, had shown improved CBH activity with less than impressive improvement in the other two cellulases tested. To obtain an overall picture of changed cellulase activities in the various strains, the results illustrated in Figures 3.1, 3.2 and 3.3 have been summarised in Table 3.1.

Table 3.1: Enzyme activity across all strains following a 72 hour incubation and post 48 hours of cellulose conversion as compared to the parental strains. *Percentages are an indication of improvements or reductions in activity after expression of the gene indicated, when compared to the parental strains. Green cells represent an increase that had exceeded 10%, while red is an indication of a reduction in activity exceeding 10%.*

Strain:	Assay:	*BET1*	*PSE1*	*SEC9*	*SEC18*	*SED5*	*SNC1*	*SSO1*
ECC	BGL	-10%	-17%	-12%	-15%	+46%	-27%	-21%
	EG	+87%	+72%	+14%	+50%	+231%	+5%	+36%
	CBH	+101%	+45%	+46%	+16%	+61%	-1%	+9%
	Avicel conv.	-4%	+84%	+95%	-16%	+59%	+0,05%	-14%
A26	BGL	+99%	+20%	+39%	+95%	+23%	+20%	+48%
	EG	-28%	-52%	-14%	-45%	-74%	-59%	-45%
	CBH	+25%	-22%	-15%	+30%	+88%	-33%	+20%
	Avicel conv.	-33%	-33%	-7%	-13%	+186%	-36%	+40%

The ECC based strains showed improvements across a greater number of the co-expressed SNARE proteins and were enhanced to a greater extent compared to the ΔA26 strains expressing the same genes. As mentioned, this may be due to the different copy number and inherent differences in the activities of the various cellulases in these two parental strains. While it should be noted that there is no one SNARE protein that can be overexpressed in an effort to improve all cellulase activity in a specific strain, the overexpression of SED5 showed the most robust improvement across all of the strains tested in this study. The perhaps unexpected result that improvement of some cellulases did not lead to overall improvement in cellulose (avicel) conversion, is likely due to incorrect ratios of the cellulases that may have been caused by our rational engineering. Transformants displaying relatively high cellulase activity levels were selected for further growth analysis as high cellulase activity often affects the growth of the yeast (Van Zyl *et al.* 2016).

3.3. Growth Analysis

The strain growth data seen in Figure 3.4 was obtained by growing a pure culture of each strain in YPD media, agitated at 180rpm for 39 hours at 30˚C. The growth kinetics of the previously

selected superior performing strains were determined, to ascertain the effects of these additions when compared to the parental strains.

Growth of the three best performing BYBGSS, ECC and ΔA26 based strains transformed with secretion enhancement genes along with their parental strains was tested at various time points for a total of 39 hours and the OD_{600} of each strain was plotted. The strains tested showed very little decrease in growth capability when compared to the parental strains that had been subjected to identical growth conditions. Moreover, in most cases we observed an increase in the highest OD_{600} levels attained. The only exception to this statement would be strains ΔA26_SSO1 and ECC_SED5. While ΔA26_SSO1 showed a small degree of reduction in growth towards the very end of the cultivation period, ECC_SED5 displayed a significant reduction in growth after just 8 hours and continued to grow at a reduced level when compared to the ECC parental strain. The BYBGSS_PSE1 transformed strain (Figure 3.4.A) reached a higher OD_{600} reading of 15.98 when compared to the parental strain which had an OD_{600} of 14.64. BYBGSS_PSE1 and BYBGSS_SED5 showed comparable growth patterns, reaching 15.93, while BYBGSS_SEC9 surpassed both strains, reaching an OD_{600} of 18.56 by 39 hours.

A dissimilar pattern was observed for the ECC transformed strains (Figure 3.4.B). The ECC_PSE1 strain reached an OD_{600} of 38.66 and ECC_SEC9 an OD_{600} of 29.49 surpassing the parental strain's OD_{600} of 20.63 while the OD_{600} of ECC_SED5 trailed behind with an OD_{600} of 12.94. The ECC_SED5 strain began with a growth rate below the parental strain and never increased above any of the strains. This may be correlated to its higher cellulase activities when compared to the other strains (Figure 3.2 A-C), possibly representing a metabolic burden on the cells (Van Rensburg *et al*. 2012). The improvement of fitness in some criteria can sometimes lead to reduced fitness for other circumstances (Liu, *et al*., 2014; Kroukamp, *et al*., 2017). The many possible contributing factors leading to this reduction in growth rate include the cellular stress associated with heterologous protein folding, degradation of misfolded protein in the ER, as well as the competition for resources for the synthesis of native proteins and heterologous enzymes (Liu, *et al*., 2014). There have been numerous attempts to reduce the metabolic burdens in yeast. An example of this includes the over-expression of chaperone proteins (Liu, *et al*., 2017). One such chaperone is Pdi, which inhibits the aggregation of misfolded proteins, and is part of at least two

multienzyme complexes (Tsygankov & Padkina, 2018), while BiP, another such chaperone, aids in protein folding, stabilizing the protein and preventing it from aggregating with other unfolded/misfolded proteins (Smith, *et al*., 2004).

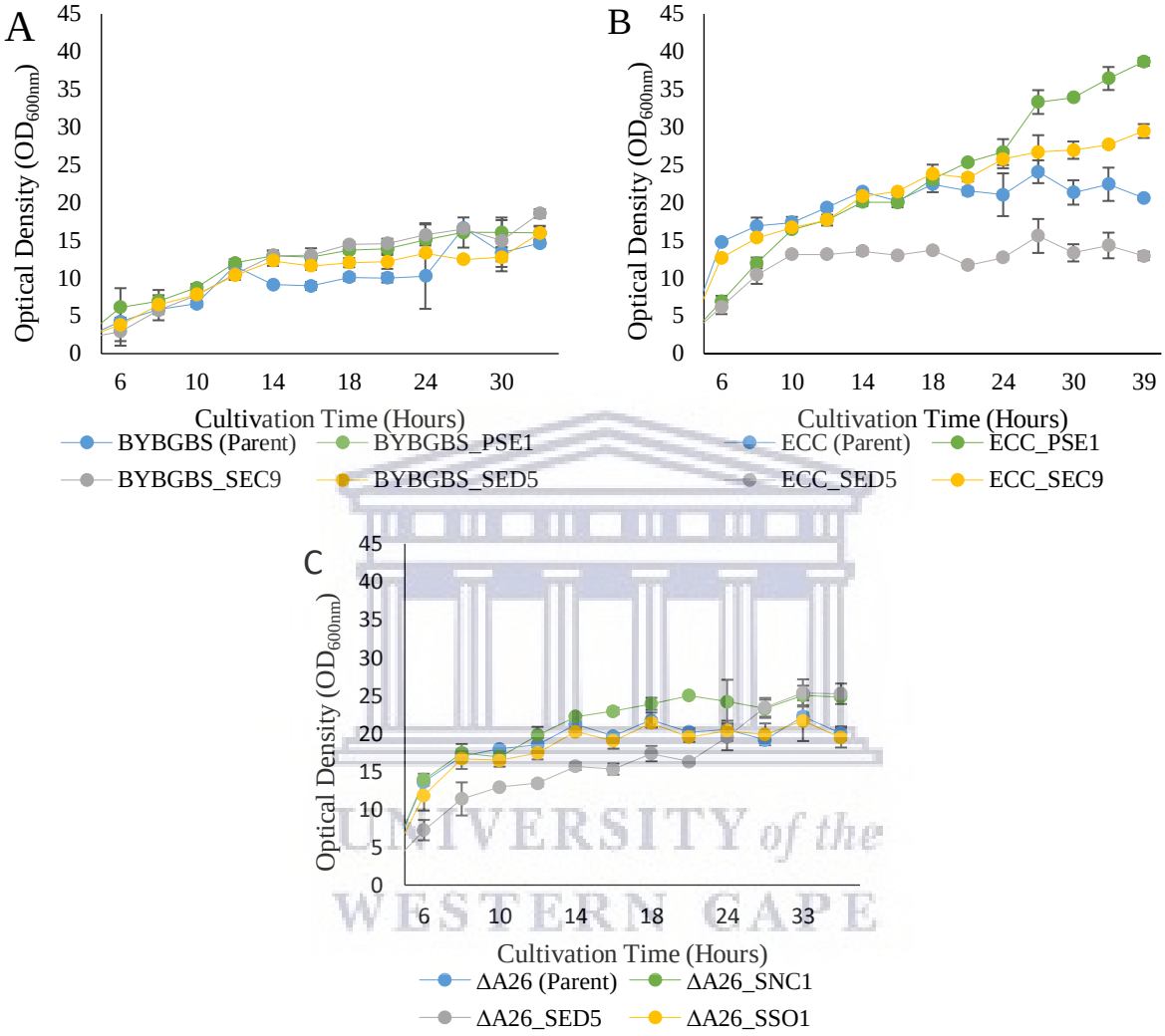

Figure 3.4: (A) Growth kinetics of BYBGSS parental strain and recombinant BYBGSS strains transformed with PSE1, SEC9 or SED5 SNARE genes. (B) Growth kinetics of ECC parental strain and recombinant ECC strains transformed with PSE1, SEC9 or SED5 SNARE genes. (C) Growth kinetics of ΔA26 parental strain and recombinant ΔA26 strains transformed with SNC1, SSO1 or SED5 SNARE genes. *Stains used had been identified to have superior enzyme activities over all other transformants. Strains were cultivated over a period of 39 hours in yeast peptone dextrose media and absorbance recorded at 600nm (OD_{600}). All values represent mean values of cultures grown in triplicate with error bars indicating the standard deviation from the mean value for each strain.*

For the ΔA26 based strains, ΔA26_SNC1 and ΔA26_SED5 both showed an increase in maximum OD attained with an OD_{600} of 24.83 and 25.23, respectively by 39 hours when compared to an OD_{600} of 20.01 for the parental strain, while ΔA26_SSO1 showed a slight decrease, measuring an

average OD_{600} of 19.43. After seeing the increased cellulase activity of ΔA26_SED5, as well as its improvement in cellulose conversion rates, one might have expected that this strain would show greater signs of metabolic burden in the form of reduced cell growth, especially in the light of the poor growth of ECC_SED5. However, after 24 hours ΔA26_SED5 was able to show excellent growth, surpassing the growth of all other strains despite its slower start. The observation that growth kinetics of the ECC_SED5 and ΔA26_SED5 are contrary, despite both displaying increased levels of cellulase secretion is perplexing. Due to the *SED5* gene being integrated in both of these strains, similar physiological effects might be expected. However, these transformations rely on delta integrations, which would likely occur in different loci in the genome. It is thus conceivable that the integration caused differential expression patterns of genes surrounding the locus of integration, which may have led to the differences in observed growth phenotype. Such differences due to contrasting integration loci of the same gene has been reported previously. Using red fluorescence protein (RFP), Wu and co-workers were able to confirm that positions of integration had varied greatly and the distribution of these RFPs was roughly random, except near the telomere and centromere regions (Wu, *et al*., 2017). A study by van Zyl and colleagues (2014) showed that a strain in which they had overexpressed *SSO1* and simultaneously overexpressed the *SNC1*, *SSO1* and *SEC9* displayed diminished growth after 29 hours, similar to that of ΔA26_SSO1 that showed a decrease in overall growth rate in this study.

3.4. Fermentation Derived Stress Tolerance Testing

During the production of bioethanol, yeasts are often subjected to varying concentrations of ethanol, high temperatures, osmotic stress and the presence of lignocellulosic-derived inhibitors, often in succession or in combination in the fermentation media (Mukherjee, *et al*., 2014). A major challenge in rational engineering to improve CBP host strains is to increase the level of productivity without increasing the metabolic burden, lowering the tolerance capabilities of the yeast strain, or creating possible redox imbalances and product inhibition (Davison, *et al*., 2020; Deparis, *et al*., 2017). The production of compounds via recombinant pathways have been identified to be more sensitive to stressors than the wild-type pathways (Deparis, *et al*., 2017). An example of this would be an increased sensitivity towards ethanol and osmotic stress in *S. cerevisiae* strains co-expressing the exocytic SNARE genes and heterologous cellulase genes even though they had shown higher cellulase production (Van Zyl, *et al*., 2014, 2016).

A study by Ilmén and co-workers (2011), found that while some proteins may be closely related in sequence and structure, their expression induced varying levels of UPR induction, leading to contrasting levels of cellulases being secreted. One can therefore assume that the stress experienced by the host cell as a result of different heterologous proteins is due to these proteins affecting different points within the gene transcription, translation, and maturation processes. Aside from this, environmental stressors also need to be considered. Abrupt changes in the environment requires a quick adjustment of the yeast internal machinery for adequate growth in the changing environment (Davison, *et al*., 2016).

In order to ascertain if the secretion enhancement genes transformed into these strains had any negative effects on the tolerance capabilities towards various environmental stresses, ten-fold serial dilutions of these strains were spotted, together with the parental strains on YPD media containing the appropriate inhibitors (Figure 3.5). Most strains were unaffected by the addition of the SNARE protein that was overexpressed in each strain when introduced to the various fermentation stressors in comparison to their relevant parental strains. Some sensitivity towards increased levels of ethanol was noted for BYBGSS_PSE1 and ΔA26_SED5. Surprisingly, a few of these strains showed an increased resistance to some of the fermentation stressors, namely increased resistance to ER stress by many of the transformant strains, as well as a pronounced increased tolerance to osmotic stress seen in BYBGSS_PSE1.

3.4.1. Heat Tolerance

Optimal growth temperature of an organism allows for its fastest growth rate but may not directly translate to optimal protein yield (van Zyl, *et al*., 2014). Temperature has the ability to significantly affect cell metabolism by affecting the regulation and abundance of ER foldases and chaperones, as well as many other proteins involved in stress response and protein processing. To assess the tolerance of the strains transformed to contain the relevant SNARE proteins to higher temperatures, ten-fold serial dilutions of these strains were grown at 35°C on YPD media for a total of 48 hours together with the parental strains. Plates incubated at 30˚C were included as a control condition (Figure 3.5). After exposure to the two temperature conditions, most strains were unaffected by the change in temperature. It was, however noted that the ECC_SED5 strain grew poorer than the parental strain at both 30˚C and 35˚C. The ECC_SED5 strain had significantly higher enzyme activities, especially endoglucanase activity, inferring greater metabolic burden and secretion stress in this strain. A slight increase in the growth for strain ΔA26_SEC9 was also

observed at both 30˚C and 35˚C when compared to the ΔA26 parental strain, indicating that the addition of the SEC9 SNARE for this strain may have increased its overall resistance to higher temperatures.

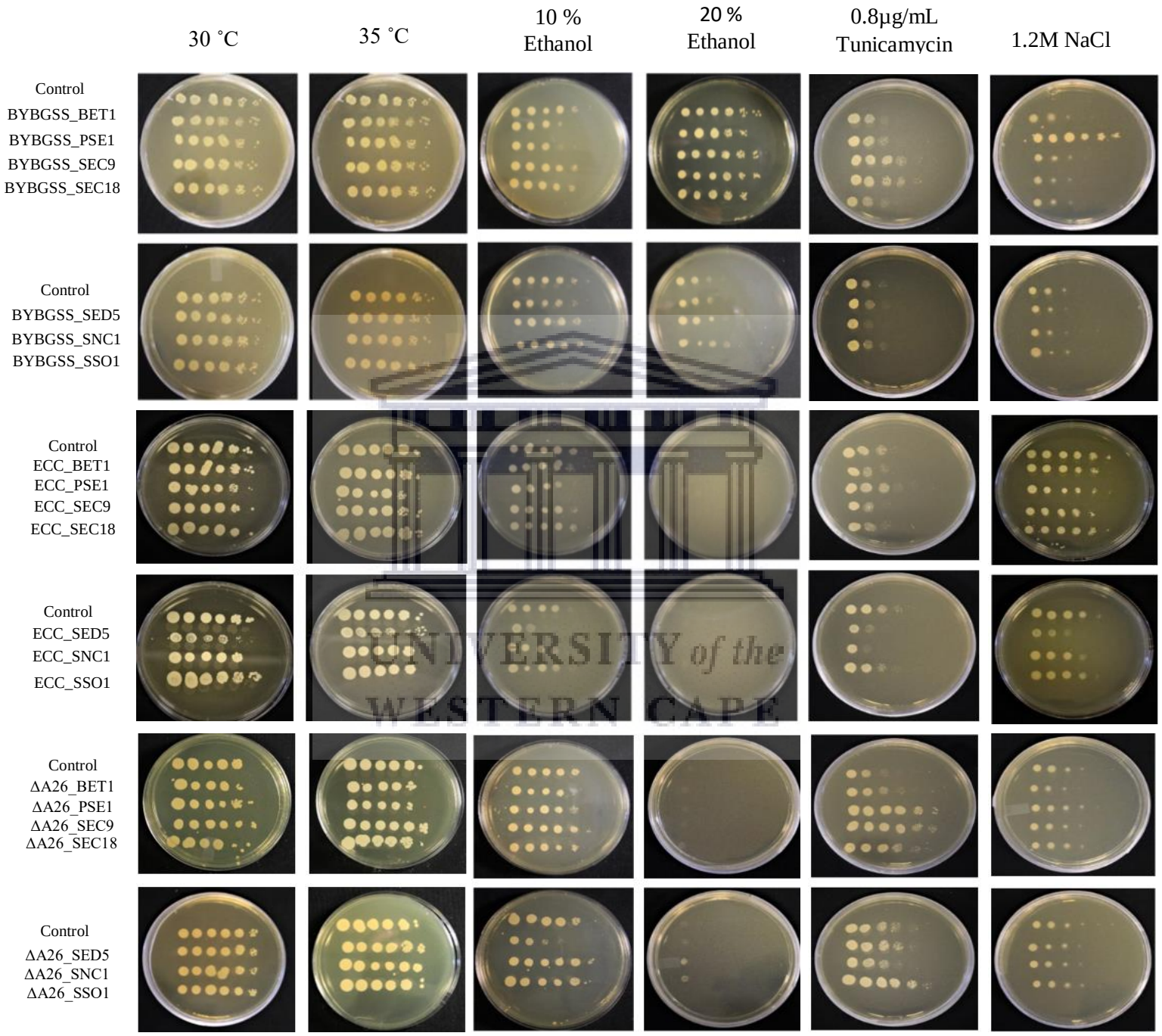

Figure 3.5: Stress plate assays to test for fermentation associated stresses after 48 hours of cultivation for BYBGSS, ECC strains and ΔA26 transformant strains. *All cultures were standardized to an OD_{600nm} of 1.4 after which 3µL of tenfold serial dilutions of the cultures were spotted on yeast peptone dextrose agar supplemented with various stressors as indicated.*

3.4.2. End Product Inhibition

For the distillation of the fermentation end product ethanol to be considered economically viable, a minimal concentration of 4-5% (v/v) ethanol is required for use in the industrial sector (Deparis, *et al*., 2017). This is because the demand for energy during the distillation process increases dramatically when lower ethanol concentrations are observed. Higher ethanol yields and titres have become more obtainable with the advancement of the production process and for this reason, yeast strains with higher maximal ethanol accumulation capacity are required for use in industry. Even at low concentrations, ethanol inhibits the growth of yeast by inhibiting cell division, decreasing the cell volume and specific growth rate. High ethanol concentrations on the other hand are capable of reducing the cell viability, as well as increasing cell death rates (Stanley, *et al*., 2010). The cell metabolism and biosynthesis of macromolecules are also negatively impacted by ethanol due to the production of heat shock-like proteins, it may also result in a lower rate of RNA and protein accumulation, or denature intracellular proteins and glycolytic enzymes, or reduce their activity (Hu, *et al*., 2007). While CBP yeasts could drive the cost of LCB bioconversion down, any strain engineering to improve cellulase secretion cannot do so at the cost of ethanol productivity and tolerance due to the economic consequences.

To ensure the transformed *S. cerevisiae* strains used in this study were able to grow in the presence of ethanol after the addition of the various SNARE proteins, 10-fold serial dilutions of each strain and the relative parental strain were spot plated on YPD media supplemented with 10%, 20% and 30% (v/v) ethanol and incubated at 30˚C for 48 hours (Figure 3.5). Plates supplemented with 10% ethanol showed that most BYBGSS based strains were unaffected by the addition of their relevant SNARE proteins. All strains were able to grow on 10% ethanol, however BYBGSS_BET1, BYBGSS_PSE1 and BYBGSS_SED5 displayed slightly increased sensitivity to the 10% ethanol. A similar result was noted in a previous study where strains that had overexpressed the SED5 SNARE protein had increased sensitivity towards ethanol as well as decreased osmotic tolerance (van Zyl, *et al*., 2016). At 20% ethanol concentrations, BYBGSS strains appeared to be mostly unaffected, with a slight sensitivity displayed by BYBGSS_BET1 and BYBGSS_SEC18. It also appeared that BYBGSS_SSO1 may have gained some ethanol resistance, as an increased colony growth was observed, even after several serial dilutions, when compared to that of the parental strain. Growth competence was almost completely diminished at a 30% ethanol concentration for the BYBGSS strains (data not shown).

ECC based strains were almost completely unaffected when plated on media supplemented with 10% ethanol. The ECC_SED5 strain was an exception to this. However, this strain had previously shown a compromised growth phenotype in comparison to the parental strain when grown at optimal conditions (Figure 3.5). Growth competence of all ECC strains, including the parental strains were completely diminished after introduction to a 20% ethanol concentration. The same trend was seen for the ΔA26 based strains, including the parental strain, consistent with the results reported by van Zyl and collaborators (2016). The ΔA26_SNC1 and ΔA26_SSO1 strains did, however, show a small ability to grow under the stress of 20% ethanol but growth had been retarded, when compared to the growth displayed at 10% concentrations.

BYBGSS based strains were notably less affected by ethanol concentrations at 20% compared to ECC and ΔA26 based strains that displayed less resistance to ethanol, showing that the expression of the additional cellulases (EG and CBHs) were a greater factor in this sensitivity than the overproduced SNARE proteins. The growth of the transformant strains closely mimicked that of their parental strains inferring that the addition of these SNARE proteins had little to no effect on the growth competency of these strains under ethanol stress, with the exception of SED5. Increased metabolic burden due to greater cellulase production had likely led to the increased sensitivity to ethanol.

3.4.3. ER Stress Tolerance

Tunicamycin is a bacterial toxin that inhibits *N*-linked glycosylation of nascent polypeptides in the ER (Bull & Thiede, 2012). Tunicamycin thus triggers ER stress responses, such as the unfolded protein response (UPR) through an accumulation of improperly folded proteins and hinders efficient protein secretion in eukaryotes. It can thus be used as a means of UPR induction, effectively causing ER stress in eukaryotic cells. The resistance of a strain to tunicamycin can be used as an indication of how much ER-stress it may be experiencing (Davison, *et al*., 2019). In order to investigate any observable changes to ER stress tolerance in the transformed *S. cerevisiae* strains used in this study, 0.5 and 0.8µg/mL tunicamycin was supplemented into YPD media onto which ten-fold serial dilutions of each strain, together with the parental strains were spot plated and incubated at 30˚C for 48 hours (Figure 3.5).

When the BYBGSS and ECC based strains were grown in the presence of 0.8µg/mL (data for 0.5µg/mL not shown) of tunicamycin, it was noted that while none of the strains showed any

reduction in tolerance to ER stress, there was an increase in tolerance across all the BYBGSS transformants, excluding BYBGSS_SNC1. An increased sensitivity towards the mimicked ER stress was seen for ECC_SED5 and ECC_SNC1. A large increase in growth competency was noted for ECC_PSE1 as well as BYBGSS_PSE1 and BYBGSS_SEC9.

Interestingly, an increase in tunicamycin resistance was noted for many of the ΔA26 based strains when compared to that of the parental strain, namely strains ΔA26_PSE1, ΔA26_SEC9, ΔA26_SEC18 and ΔA26_SSO1. However, ΔA26_SNC1 displayed reduced growth competency, while ΔA26_BET1, and ΔA26_SED5 seemed to be unchanged following the addition of these SNARE proteins. The increased tunicamycin tolerance observed in strains that produce secretion enhancement proteins is likely linked to the mechanism by which these proteins enhance cellulase secretion. Improvement of the secretion pathway through expression of these proteins leads to better secretion competence, which also decreases the amount of observed ER-stress (Davison *et al*. 2019). It is, however, worth noting that across all of the strains, those that had been transformed to overexpress *SNC1* had shown increased sensitivity towards the mimicked ER stress, indicating that this SNARE protein may be further increasing the ER stress in these cells.

3.4.4. Osmotic Tolerance

High sugar and salt concentrations cause osmotic stress during the production of bioethanol and is one of the most common stresses that industrial fermentation organisms have to contend with (Deparis, *et al*., 2017). The pretreatment methods, or procedures undertaken to detoxify the hydrolysate during 2G bioethanol production could also negatively affect the fermentation rate and yield of the yeast due to accumulation of salts. Due to the costs involved, removal of these salts is not an economically viable option. The need to achieve high product titres requires the use of high gravity fermentations, however, this process is known to increase osmotic pressure (Bai & Zhao, 2011). To test the osmotic tolerance of the transformed *S. cerevisiae* strains used in this study, 1M and 1.2M NaCl was supplemented into YPD media onto which ten-fold serial dilutions of each strain, together with the parental strains were spot platted and incubated at 30˚C for 48 hours (Figure 3.5 – only 1.2M NaCl resistance is shown).

The BYBGSS based strains seemed mostly unaffected following the overexpression of the SNARE proteins. When compared to the parental strain, an increase in osmotic tolerance was observed in BYBGSS_BET1. In contrast, previous studies using a different BGL encoding gene

have reported a decrease in the cell's tolerance to osmotic stress due to the overexpression of this SNARE protein (van Zyl, *et al*., 2016). This difference in BGL encoding genes may, however, be the reason for these contradictory findings.

The ECC and ΔA26 based transformants showed no change in the resistance to osmotic stress after exposure to 1M and 1.2M NaCl with the exception of the growth compromised ECC_SED5, where a slight decrease in the growth competency was observed. This coincides with the results seen during the testing of its ethanol resistance further substantiating the results reported by Van Zyl and co-workers (2016).

3.5. Fermentation of Crystalline Cellulose

To test if the improved cellulase secretion and stress tolerance phenotypes engineered into the strains detailed above translated to improved bioconversion of crystalline cellulose, small-scale fermentations of 10g/L avicel were performed and HPLC results of the end point concentrations of these fermentations are tabled below (Table 3.2). Yeast cells are often subjected to a variety of stress conditions throughout the course of the fermentation process and are required to adapt to the changing environment in order for the cells to proliferate, even in the presence of the fermentation derived stressors (Bai & Zhao, 2011). An example of the cell responding to various fermentative stressors is the cell producing glycerol to act as an osmotic stabiliser, while yeast growth is impeded by the acetic acid also released by the cell, resulting in the inhibition of the fermentation process and limits the process productivity.

After 96 hours of fermentation, the ethanol concentrations appeared to be mostly unaffected by the addition of these SNARE proteins. However, ECC_PSE1 showed a slight increase of 11% to 2,41g/L compared to 2,18g/L displayed by the ECC parental strain while the ΔA26 transformant strains had shown a decrease of roughly 8% when compared to the ΔA26 parental strain which attained similar levels of ethanol production as was previously reported (Liu, *et al*., 2017). Based on the improved avicel conversion reported in Figure 3.3 and the general lack of growth differences between the parental and SNARE overexpressing strains in stress assays (Figure 3.5), we expected improved levels of ethanol production for these strains during avicel fermentation. A higher cell loading to avicel ratio should be attempted in future to attain higher conversion levels.

Table 3.2: Composition of 96-h fermentation broth samples detected by high-performance liquid chromatography. *The parental ECC and ΔA26 strain were included to be used as reference. Baseline levels were determined for each sample and subtracted to achieve results listed. All values represent mean values in g/L of the fermentations conducted in triplicate after 96 hours. Numbers in brackets represents the standard deviation from the mean value of each sample.*

Strain	Cellobiose	Glucose	Glycerol	Acetic Acid	Ethanol
ECC (Parent)	0,322 (0,279)	0 (0)	0,417 (0,007)	0,863 (0,050)	2,175 (0,049)
ECC_PSE1	0,321 (0,278)	0 (0)	0,414 (0,008)	0,825 (0,100)	2,413 (0,021)
ECC_SED5	0,000 (0,000)	0 (0)	0,408 (0,001)	0,667 (0,006)	1,807 (0,004)
ECC_SEC9	0,483 (0,001)	0 (0)	0,407 (0,002)	0,813 (0,009)	2,209 (0,008)
δA26 (Parent)	0,322 (0,279)	0 (0)	0,403 (0,009)	0,813 (0,043)	2,338 (0,048)
δA26_SNC1	0,161 (0,278)	0 (0)	0,380 (0,011)	0,777 (0,033)	2,171 (0,096)
δA26_SED5	0,160 (0,278)	0 (0)	0,377 (0,011)	0,711 (0,012)	2,140 (0,036)
δA26_SSO1	0,160 (0,278)	0 (0)	0,421 (0,004)	0,785 (0,011)	2,112 (0,018)

It was noted that most strains had adequately used the available cellobiose in the media as low levels of this was detected across all but one of the strains. All available glucose was used up by all of the strains tested and glycerol levels remained low. The acetic acid levels had not exceeded that of the parental strains for any of the strains and ethanol concentration did not appear to be negatively affected across most of the strains, excluding ECC_SED5, which showed a reduced concentration of ethanol after the fermentation period. This might be related to the known growth defect previously observed for this strain.

An earlier study showed that the conversion of cellobiose to glucose is a limiting step in cellulosic SSF (Tang, *et al.*, 2013). For this reason, increased capabilities of cellobiose fermentation could enhance the efficiency of the cellulose hydrolysis in *S. cerevisiae*. Cellulases are often strongly inhibited by the accumulation of cellobiose, thereby affecting the degradation of the cellulose and consequently ethanol production. While cellobiose levels remained at relatively low concentrations for the duration of the fermentation period in both ECC and ΔA26 based strains, ECC_SEC9 displayed an elevated concentration of 0.483g/L cellobiose when compared to the parental strain (0.322 g/L) at the end of the fermentation period. All other strains, apart from ECC_PSE1 which was unchanged from the parental strain, showed a decrease in cellobiose concentrations. The greatest reduction in cellobiose was observed for ECC_SED5, having no detectable cellobiose available after 96 hours confirming improved BGL activity in this strain.

The production of glycerol in *S. cerevisiae* is inversely linked to ethanol, however, a small amount of glycerol production is necessary to help maintain an optimal redox equilibrium and proper cellular osmoregulation (Carvalho-Netto, *et al.*, 2015). Glycerol is the second primary by-product

of fermentation after cell-biomass, formed as an alternative reduced product and usually to the detriment of the ethanol production yields. For this reason, reducing the formation of glycerol may result in higher ethanol production rates (Favaro, *et al*., 2015). When compared to the 0.403g/L of glycerol produced by the parental strain, ΔA26_SNC1 and ΔA26_SED5 showed slightly reduced levels of glycerol production of 0.380g/L and 0.377g/L, respectively. All other strains remained relatively unchanged after the 96 hour fermentation had been completed. No excessively high glycerol concentrations were observed, inferring that none of the strains experienced stress causing metabolic imbalances, which could lead to the diversion of carbon away from ethanol production, resulting in lower-than-expected ethanol yields (Sakwa, *et al*., 2018). It was thus inferred that the ethanol concentrations of these strains should not be affected as a direct result of glycerol production. Acetic acid, like other organic acids, are produced in low concentrations by *S. cerevisiae* (Carvalho-Netto, *et al*., 2015), consequently the detection of organic acids in fermentations are often attributed to bacterial contaminants during the production of bioethanol. The low levels observed across all of the strains listed in Table 3.2 serves as an additional indication that no bacterial contamination had taken place during this fermentation cycle.

3.6. Gene Combination Studies

Strains that displayed the greatest improvement in cellulase activity in the ECC and ΔA26 backgrounds transformed with the relevant SNARE proteins, aside from the strains overexpressing SED5 were subsequently selected for further gene combination studies. These strains were further transformed with the *SED5* SNARE encoding gene, which was shown to yield a consistent improvement on cellulase activity in this study as well as in previous work (van Zyl *et al.*, 2016; Table 3.1). PCR confirmation was performed on all strains used in the gene combinational studies (Appendix A4-6) to ensure that each of the SNARE genes had been successfully transformed into all of the strains.

The result of the double overexpression studies (Figure 3.6) showed that BGL, EG and CBH activity had increased for the ΔA26 strains when compared to the original ΔA26 parental strain, however this same trend was not seen for the ECC double overexpression strains. While these strains were able to display an improvement of BGL, EG, and CBH activity over the parental strain, these results did not always exceed that of the single overexpression strains to which the SED5 protein was added. The ΔA26_SSO1_SED5 strain, however, showed improvement across

two of the three cellulases tested with little-to-no change seen for the third cellulase, while too minute a change in the cellulase activity was seen for any of the ECC transformants to infer that the additional overexpression of the SED5 SNARE protein was beneficial to this strain.

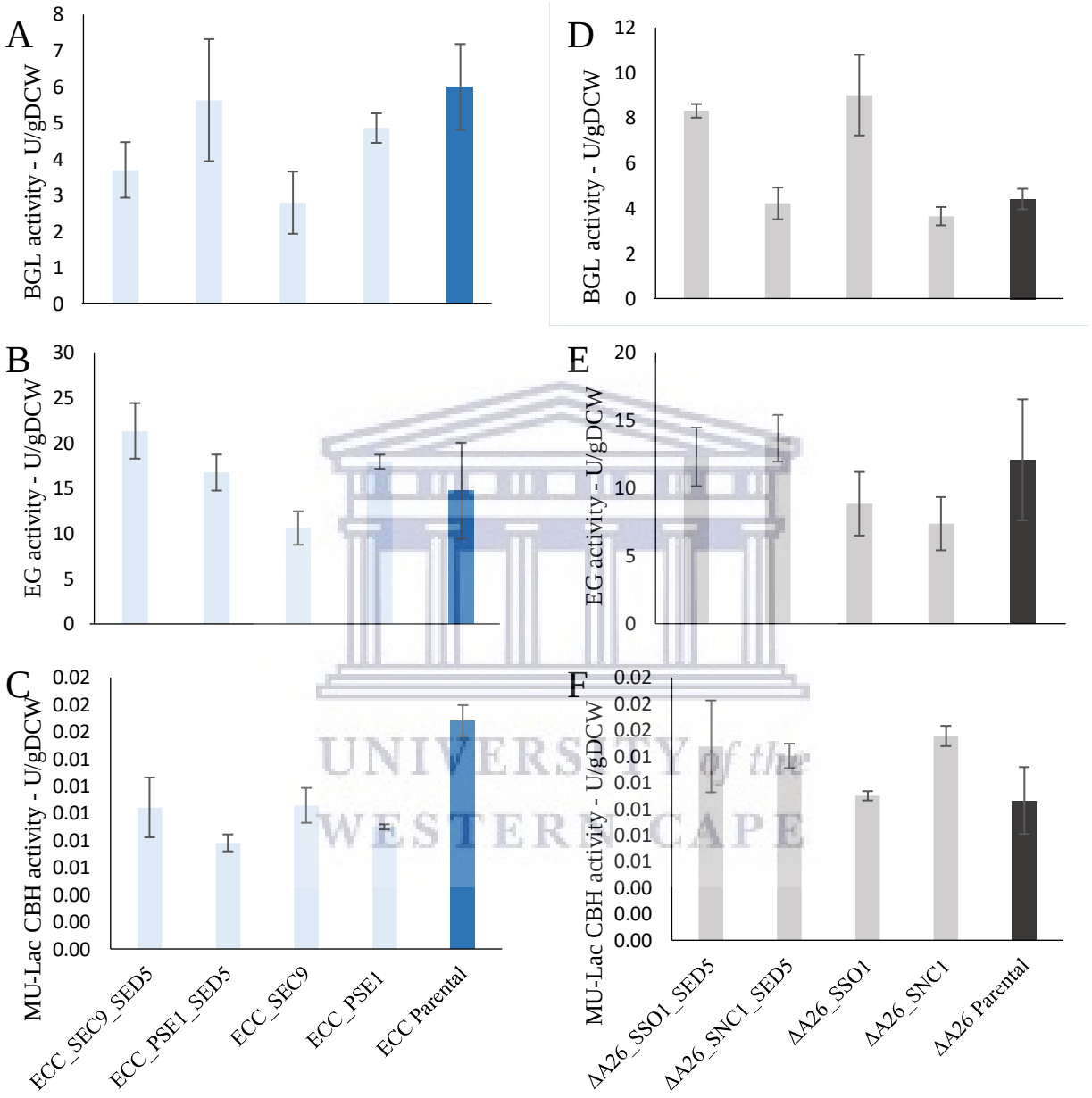

Figure 3.6: The enzyme activity profiles of the previously identified ECC strains transformed with SEC9 and PSE1 and ΔA26 strains containing SSO1 and SNC1 further transformed to overexpress the SED5 SNARE protein. (A&D) β-glucosidase activity on p-nitrophenyl-β-D-glucopyranoside. (B&E) endoglucanase activity on carboxymethyl cellulose. (C&F) cellobiohydrolase activity on 4-methylumbelliferyl-β-D-lactoside. *Values obtained were normalised using dry cell weight (DCW) of each strain and the parental ECC and ΔA26 strain was used as reference. All values represent mean values of assays conducted triplicate with error bars indicating the standard deviation from the mean value for each strain.*

Figure 3.6 (D) showed that after the 72 hours of cultivation, the ΔA26_SNC1_SED5 strain had outperformed the ΔA26_SNC1 strain, displaying a 15% increase in the BGL activity. The ΔA26_SSO1 and ΔA26_SSO1_SED5 strains displayed a 50% and 39% improvement in BGL activity when compared to the parental strain. While an improvement in activity was noted when compared to the parental strain, this represented a decrease of 11% in BGL activity after the additional overexpression of the *SED5* SNARE encoding gene to the ΔA26_SSO1 strain. Additionally, the overexpression of the SED5 SNARE protein in the ΔA26_SNC1 strain was unable to improve BGL activity. Figure 3.6 (A) showed that when ECC_SEC9_SED5 was evaluated against ECC_SEC9, an increase of 33% in BGL activity was observed after the 72 hour incubation period. An increase of 16% of this same cellulase was also seen for ECC_PSE1_SED5 when compared to the ECC_PSE1 strain.

The ΔA26_SSO1_SED5 strain outperformed the EG activity production of the ΔA26 parental strain, as well as the ΔA26_SSO1 strain from which this combinatorial strain was constructed (Figure 3.6 (E)). It was also noted that the ΔA26_SNC1_SED5 strain produced 39% higher EG activity than that of its own ΔA26_SNC1 parent as well as the ΔA26 parental strain. The ECC based strains that had been subjected to the double transformation appeared to both outperform the original ECC parental strain, showing an improvement of 45% and 14% for ECC_SEC9_SED5 and ECC_PSE1_SED5, respectively. However, ECC_PSE1_SED5 did not perform at a level above its ECC_PSE1 parent strain.

After 72 hours of cultivation, both the initial transformants ΔA26_SSO1 and ΔA26_SNC1 outperformed CBH activity of the original parental strain. Furthermore, ΔA26_SSO1_SED5 displayed a further improvement in CBH activity of 34% when compared to the ΔA26_SSO1 strain and 40% improvement when compared to the parental strain. It was previously reported that when SED5 was overexpressed together with SSO1, an improvement of 130% in the BGL activity was observed, however this same study also noted no improvement for the CBH activity with this gene combination in strains expressing only one of the two cellulases each (van Zyl, *et al.*, 2016). While our results coincide with the earlier study on the BGL activity, our strain showed a 32% increase for CBH activity with co-expression of these SNARE proteins. Differences in the strain backgrounds may help explain these observations. For the ECC based strains, no improvements of CBH activity with co-overexpression of *SED5* was observed.

These results do not provide a full picture of the possible improvements that may have occurred within the double transformed strains and more testing on these strains may be required to know how this further affected the growth capabilities, resistance to fermentation stressors, as well as ethanol production. The ΔA26_SSO1_SED5 strain appears to be the most promising of the strains tested for improvements in cellulase activity during these combinational studies. Our results concur with previous studies where it was shown that rational engineering strategies to improve heterologous secretion yielded positive effects but that these were protein-specific and in many cases not synergistic or even cumulative (de Ruijter, *et al.*, 2016). Evidence suggests that the overexpression of one secretory pathway gene may lead to the upregulation of other genes also involved in the secretion process. For example, Wu and colleagues (2017) reported that overexpression of the *SNC1* gene in *T. reesei* also increased *HAC1* transcript levels, while overexpression of *BIP1* upregulated *SNC1* transcription. The overexpression of one gene that may have a positive effect, may therefore mask the effect of another a simultaneously overexpressed gene, which would have been upregulated by the yeast in any case.

4. Chapter 4: Summary and Conclusion

An increasing demand for energy to keep up with the growing population has led to the exploitation of fossil resources as a fuel source. Aside from its inevitable extinction, fossil resources are also the cause of many negative environmental and economic issues (Olguin-Maciel, *et al*., 2020). The 2G biofuels that have been derived from LCB have been identified as a promising alternative to petroleum-based fossil fuels (Saini, *et al*., 2015). This method makes use of agricultural residues and waste products, providing environmentally friendly fuel substitutes, and advancements in the field will lead to this being a feasible technology to achieve energy security in future. The 1G biofuels represent well-established industries which annually produce more than 100 billion litres of ethanol (Favaro, *et al*., 2019). Advancements in 2G biofuel production was expected to allow it to overtake 1G biofuels within the next decade, however, issues such as feedstock sourcing, pretreatment, high enzyme costs and harsh fermentation conditions remain persistent problems with this technology. A more robust and thermotolerant microbial catalyst for direct microbial conversion of the substrate and with higher production efficiency and product quality would prove beneficial to the overall process. Significant efforts have thus been dedicated to expanding the substrate range and improving the robustness of 1G yeast strains for use in 2G biofuel production.

Bioethanol by means of CBP production is the intended end-goal as a bioprocessing method as it only requires a single organism and the use of a single reactor (Olguin-Maciel, *et al*., 2020). This option, however, may only be considered for large-scale biofuel production if a fit-for-process organism can be engineered. Its inherent high sugar consumption rate, tolerance of high osmolality and overall process robustness makes *S. cerevisiae* an attractive option for use in industrial settings due to the multitude of stresses which microorganisms are subjected to throughout the process of bioethanol production (Gomes, *et al*., 2021). However, this yeast requires engineered cellulolytic activity to enable its use in CBP production of 2G bioethanol from LCB. Many advancements have been achieved thus far by combining beneficial traits of numerous strains using adaptation and hybridization and targeting specific traits through genetic engineering, however there is still much to be done to develop an industrial yeast strain, superior to those currently available, to overcome the limitations involved with cellulosic ethanol production (Favaro, *et al*., 2019).

To date, studies demonstrating the ability of recombinant *S. cerevisiae* to ferment cellulosic substrates to ethanol without the addition of exogenous enzymes remain scarce (den Haan, *et al*.,

2007; Inokuma, *et al*., 2017; Davison, *et al*., 2019). One study in particular involved the engineering of a strain with the use of cocktail δ-integration, generating a cell surface adhered core set of heterologous cellulases in a ratio optimised manner for hydrolysis of crystalline cellulose (Liu *et al*., 2017). Nevertheless, due to low bioconversion efficiencies, these cell-surface engineered strains were not yet feasible for industrial use (Chen, *et al*., 2018). Low secretion titres are a major drawback that needs to be addressed in current recombinant yeast strains engineered for use in CBP. In several other studies, it was shown that heterologous cellulase secretion in yeast could be significantly enhanced through rational strain engineering, including the overexpression of SNARE encoding genes (van Zyl, *et al*., 2014; van Zyl, *et al*., 2016; Kroukamp, *et al*., 2018). This study aimed to show the potential role that SNARE proteins could play in increasing the currently attainable titres for recombinant cellulases in *S. cerevisiae* by improving the amount of cell-adhered cellulase activities of recombinant *S. cerevisiae* strains through over-expression of genes encoding SNARE proteins identified in previous strain engineering studies.

From all of the strains created in this study, ΔA26_BET1 and ΔA26_SEC18 displayed the most improved levels of BGL activity when compared to that of the parental strain while ECC_SED5 had shown the highest recorded BGL activity across all of the strains. It was also noted that ECC_BET1 displayed the greatest improvement in CBH activity across all of the strains tested, while ΔA26_SED5 had shown the highest recorded CBH activity. ΔA26_SED5 also displayed the highest recorded EG activity of all strains tested while ECC_SED5 showed the greatest improvement after the addition of the *SED5* gene when compared to the ECC parental strain. This same ΔA26_SED5 strain also displayed the greatest improvement in conversion of avicel crystalline cellulose, also displaying the highest recorded cellulose hydrolysing ability across all of the strain tested in this study. The ECC_SED5 strain displayed the most vigorous improvement, having shown an improvement for all of the cellulase activities tested during this study. While we were able to show that no one specific SNARE protein was capable of improving all tested cellulase activities in a specific strain, it was noted that the overexpression of *SED5* showed the most robust improvement across all of the strains tested in this study. Furthermore, it was also observed that the ECC based strains showed much greater improvement across a greater number of the overexpressed SNARE proteins than was the case with the ΔA26 strains overexpressing those same SNARE proteins. This is likely due to differences in the strain backgrounds, due to the different ways in which the parental strains were constructed, despite the fact that they were based

on the same original parent *S. cerevisiae* BY4741 strain. Unexpectedly, the improvements in cellulase activity did not directly translate to an increase in avicel crystalline cellulose conversion capabilities. This anomaly may be a result of incorrect ratios of the cellulases, due to our rational engineering, or shortcomings in the fermentation set up.

It was noted that the best overall growth rate across all of the strains as well as the greatest improvement in growth, was for the ECC strains while only ECC_SED5 and ΔA26_SSO1 displayed reduced growth. Other than these two strains, all other strains showed an increase in growth ability after the addition of the relative SNARE proteins when compared to that of the parental strains.

Spot plate assays were performed in which media were supplemented with various fermentation derived stressors and this showed that most strains were unaffected by the addition the relevant SNARE proteins in each of these strains. Some strains showed a small reduction in resistance to ethanol concentrations above 20%, while surprisingly, most strains showed an increased resistance to tunicamycin which was used as a means to mimic ER stress in the cells. Strain BYBGSS_BET1 also showed an increased resistance to osmotic stress, while it should be noted the strain ECC_SED5 appeared most negatively affected by the addition of the SED5 SNARE protein, as it showed a reduced resistance to ethanol, ER stress, and osmotic stress despite showing the greatest improvement in cellulase activity for all of the strains. This same strain was also identified to have had a reduced growth capability when compared to the ECC parental strain.

Strains that had been identified as having improved enzyme activity and that showed no reduced resistance to fermentation derived stressor, were selected for further gene combinational studies. Overexpressing and combining genes yielded further increases in enzyme activity for some strains, most notably strain ΔA26_SSO1_SED5, that showed the greatest improvement across the strains tested here.

Our results showed that rational strain engineering techniques could be applied to improve the production levels of cell-surface adhered cellulases which lead to improved levels of avicel hydrolysis. However, great care should be taken to maintain or improve cellulase ratios and to not improve cellulase activities at the cost of ethanol productivity or strain robustness.

4.1. Future Prospects

Future work should include:

- Liquid growth studies to test changes in strain physiology: growth, ethanol tolerance, osmotic tolerance, tunicamycin resistance and other fermentation stressors for increased sensitivity and more accurate results.
- Subject strains with combined SNARE gene expression to growth curve analysis and HPLC analysis to ascertain if the further overexpression of the SED5 SNARE protein affected the growth and ethanol production capabilities of the strains.
- Perform fermentation stressors growth tests on combinational strains in both plate and liquid media form, to determine changes in strain physiology as a result of the overexpression of the additional SNARE proteins in the strains.
- Perform cellulose hydrolysis studies using pretreated cellulose material (bagasse) to further determine cellulose conversion capabilities.
- Perform RNA sequencing to evaluate the strain background on gene expression/protein activity.
- Engineer new strains to co-display xylanases in addition to the cellulases for enhanced cellulosic bioethanol production from a wider range of substrates.

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6. Appendix

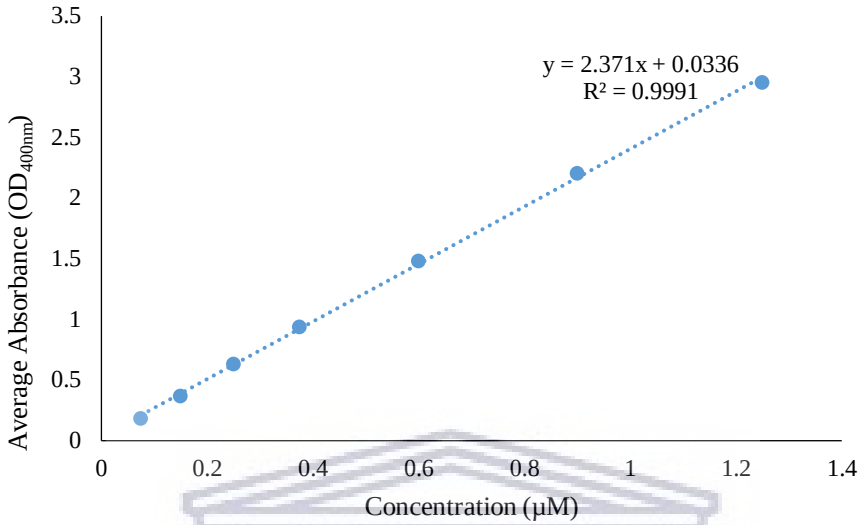


Figure A1: Calibration curve of 4-Nitrophenyl used to determine unknown concentrations of released 4-NP from which β-glucosidase activity was determined.

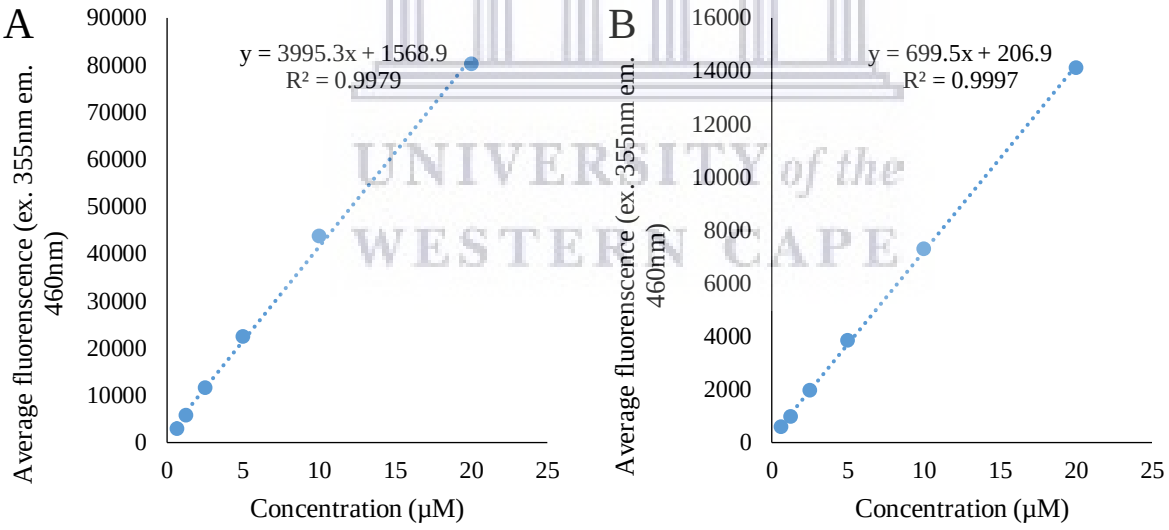


Figure A2: Calibration curve of 4-Methylumbelliferyl used to determine unknown concentrations of released methylumbelliferyl from which cellobiohydrolase activity was determined. Measurements taken using (A) clear bottom 96-well microtitre plates and (B) black bottom 96-well microtitre plates.

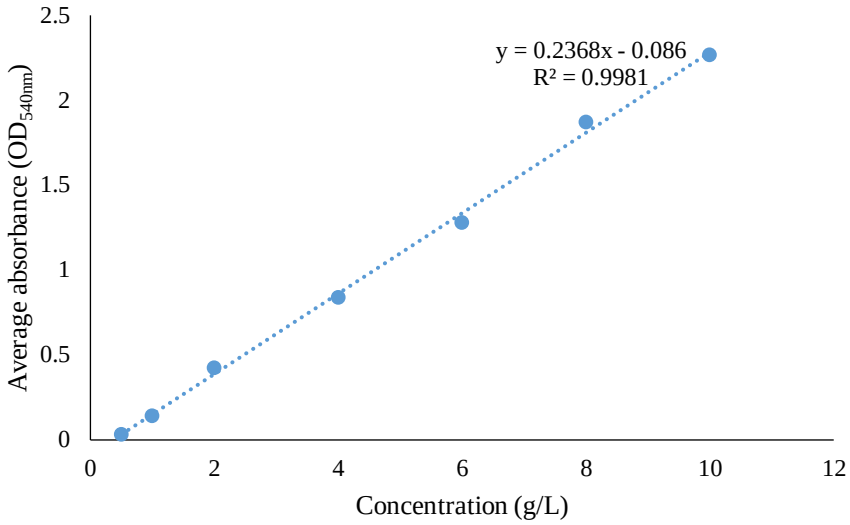


Figure A3: Calibration curve of glucose used to determine unknown concentrations of reducing sugars from which endoglucanase activity was determined.

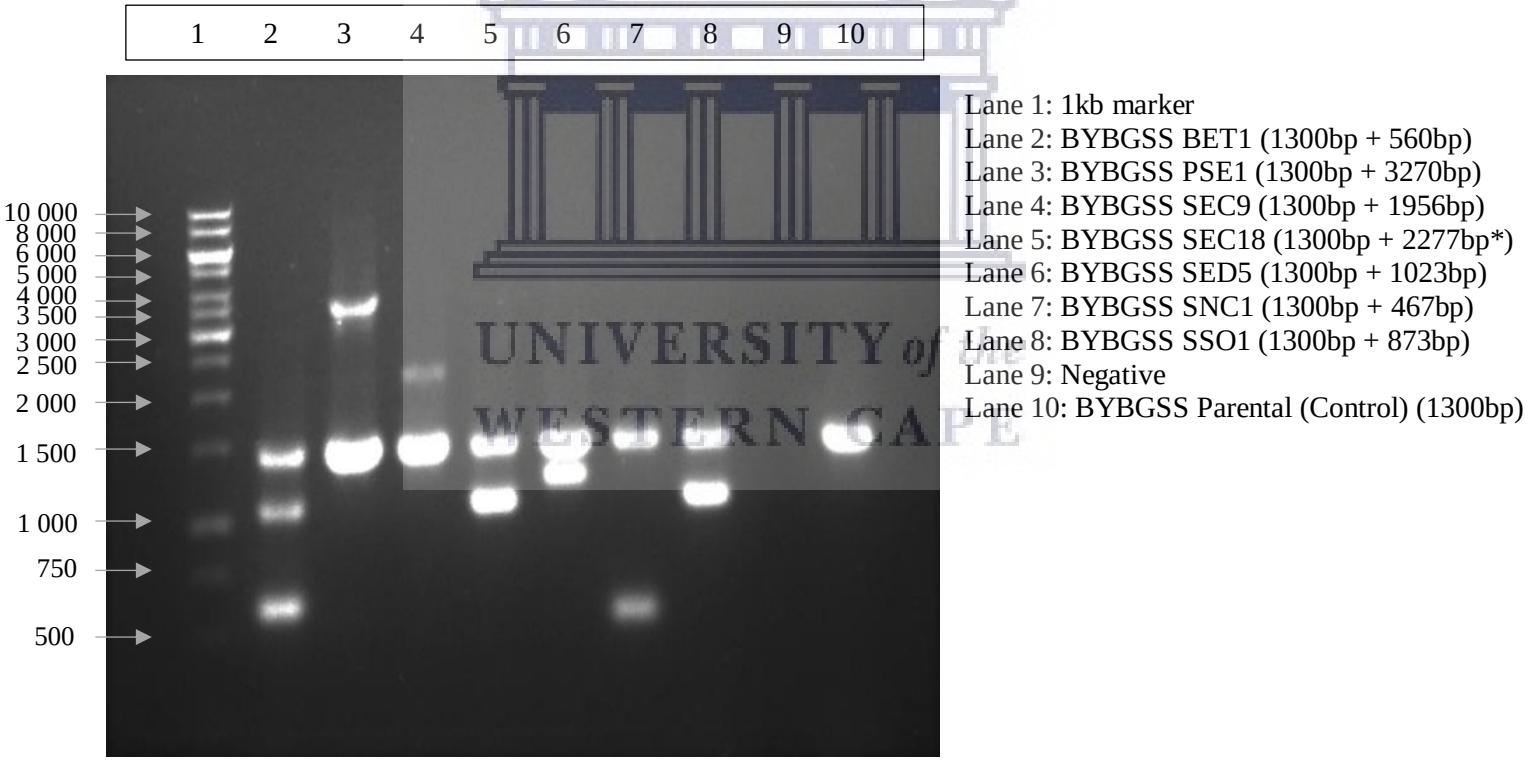
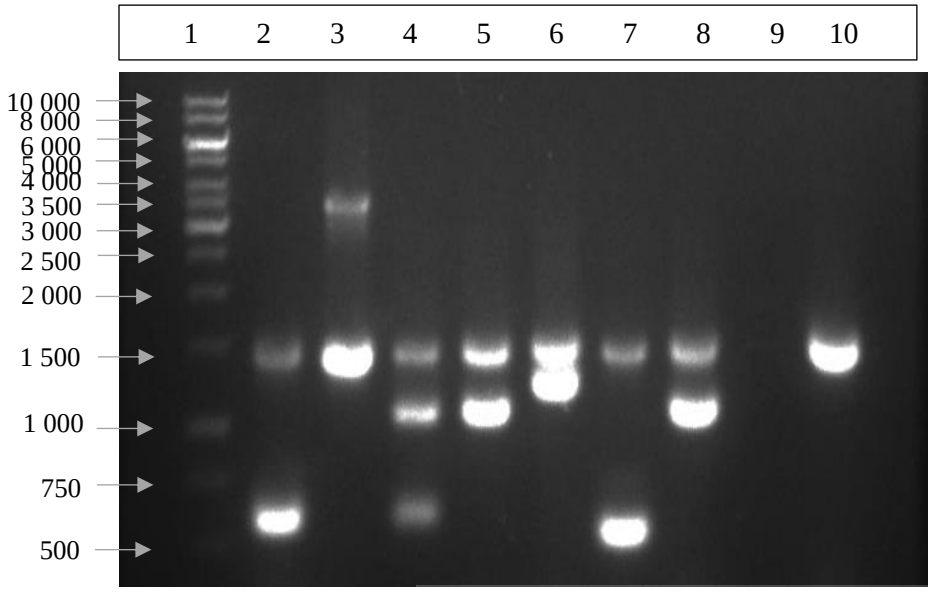
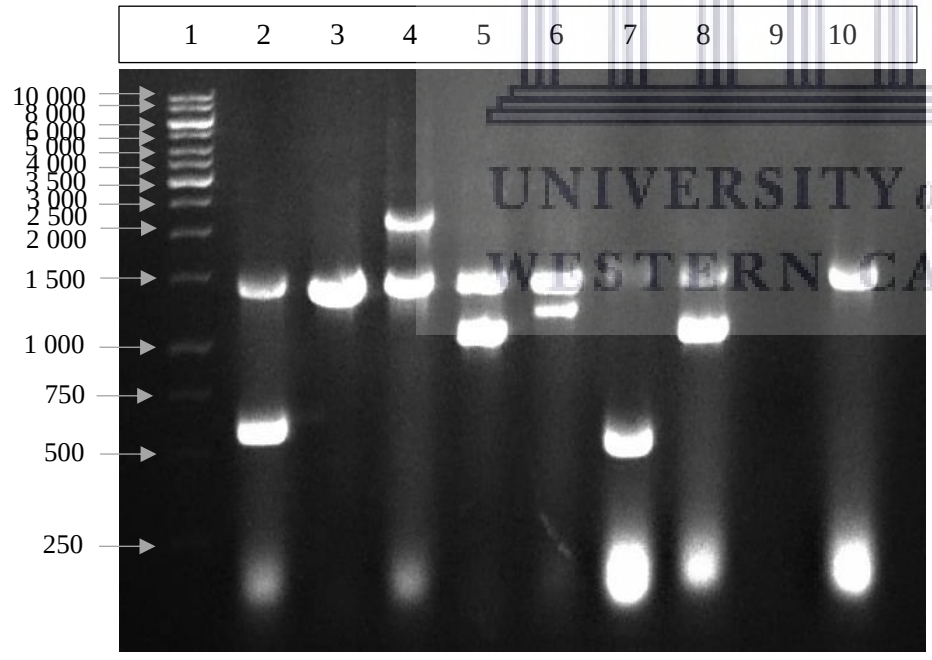


Figure A4: 1% (w/v) agarose gel electrophoresis of BYBGSS polymerase chain reaction amplified products using phosphoglycerate kinase 1-specific PCR primer sets to confirm the presence of relevant SNARE genes transformed into each BYBGSS strain. *Each lane contains the native phosphoglycerate kinase 1 gene at ~1300bp and the additional phosphoglycerate kinase 1 gene. Lane 1 shows the 1kb DNA marker, Lanes 2-8 are the BYBGSS transformants, Lane 9 is the negative control, and Lane 10 BYBGSS parental strain used as the control strain. Asterisk (*) indicates genes that had not been successfully amplified.*



Lane 1: 1kb marker
Lane 2: ECC BET1 (1300bp + 560bp)
Lane 3: ECC PSE1 (1300bp + 3270bp)
Lane 4: ECC SEC9 (1300bp + 1956bp*)
Lane 5: ECC SEC18 (1300bp + 2277bp*)
Lane 6: ECC SED5 (1300bp + 1023bp)
Lane 7: ECC SNC1 (1300bp + 467bp)
Lane 8: ECC SSO1 (1300bp + 873bp)
Lane 9: Negative
Lane 10: ECC Parent (Control) (1300bp)

Figure A5: 1% (w/v) agarose gel electrophoresis of ECC polymerase chain reaction amplified products using phosphoglycerate kinase 1-specific PCR primer sets to confirm the presence of relevant SNARE gene transformed into each ECC strain. *Each lane contains the native phosphoglycerate kinase 1 gene at ~1300bp and the additional phosphoglycerate kinase 1 gene. Lane 1 shows the 1kb DNA marker, Lanes 2-8 are the ECC transformants, Lane 9 is the negative control, and Lane 10 ECC parental strain used as the control strain. Asterisk (*) indicates genes that had not been successfully amplified.*



Lane 1: 1kb marker
Lane 2: ΔA26 BET1 (1300bp + 560bp)
Lane 3: ΔA26 PSE1 (1300bp + 3270bp*)
Lane 4: ΔA26 SEC9 (1300bp + 1956bp)
Lane 5: ΔA26 SEC18 (1300bp + 2277bp*)
Lane 6: ΔA26 SED5 (1300bp + 1023bp)
Lane 7: ΔA26 SNC1 (1300bp + 467bp)
Lane 8: ΔA26 SSO1 (1300bp + 873bp)
Lane 9: Negative
Lane 10: ΔA26 Parent (Control) (1300bp)

Figure A6: 1% (w/v) agarose gel electrophoresis of ΔA26 polymerase chain reaction amplified products using phosphoglycerate kinase 1-specific PCR primer sets to confirm the presence of relevant SNARE gene transformed into each ΔA26 strain. *Each lane contains the native phosphoglycerate kinase 1 gene at ~1300bp and the additional phosphoglycerate kinase 1 gene. Lane 1 shows the 1kb DNA marker, Lanes 2-8 are the ΔA26 transformants, Lane 9 is the negative control, and Lane 10 ΔA26 parental strain used as the control strain. Asterisk (*) indicates gene that had not been successfully amplified.*