Using CRISPR/Cas9 to construct consolidated bioprocessing strains from natural isolates of *Saccharomyces cerevisiae.*

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A thesis submitted in fulfilment of the requirements for the degree of Magister Scientiae in the Department of Biotechnology, University of the Western Cape

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I declare that "**Using CRISPR/Cas9 to construct consolidated bioprocessing strains from natural isolates of *Saccharomyces cerevisiae***" is my own work, that it has not been submitted for any degree or examination in any other university, and that all sources I have used have been indicated and acknowledge by complete reference.

Signature: 

Date: November 2022

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LIST OF ABBREVIATIONS

1G – first generation

2G – second generation

ARS – autonomous replication sequence

BGL – beta (β) glucosidase

Cas9 – CRISPR-associated endonuclease

CAZy – carbohydrate-active enzymes

CBH – cellobiohydrolase

CBM – carbohydrate-binding module

CBP – consolidated bioprocessing

CD – catalytic domain

CEN – centromeric sequence

CMC – carboxymethyl cellulose

CO_2 – carbon dioxide

CRISPR – clustered regularly interspaced short palindromic repeats

crRNA – CRISPR targeting RNA

DNA – deoxyribonucleic acid

DNS – dintrosalicylic acid

DP – degree of polymerisation

DSB – double stranded break

DTT – dithiothreitol

DCW – dry cell weight

EG – endoglucanase

ER – endoplasmic reticulum

ERAD – ER-associated degradation

FPU – filter paper unit

GHG – greenhouse gas

GH – glycoside hydrolase

GRAS – generally regarded as safe

HDR – homology-directed repair

HMF – hydroxymethylfurfural

HR – homologous recombination

HSP – heat shock protein

LB – Luria Bertani

NHEJ – non-homologous end joining

PASC – phosphoric acid swollen cellulose

PAM – protospacer adjacent motif

PCR – polymerase chain reaction

RNA – ribonucleic acid

ROS – reactive oxygen species

sgRNA – small guide RNA

SHF – separate hydrolysis and fermentation

SSF – simultaneous saccharification and fermentation

SSCF – simultaneous saccharification and co-fermentation

TFP – translational fusion partner

tracrRNA – trans-activating RNA

UPR – unfolded protein response

YCp – yeast centromeric plasmid

YEp – yeast episomal plasmid

YIp – yeast integration plasmid

YPD – yeast peptone dextrose culture media

YP – yeast peptone media

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ABSTRACT

Saccharomyces cerevisiae has gained much attention as a host for cellulosic bioethanol production using consolidated bioprocessing (CBP) methodologies, due to its high ethanol producing titres, heterologous protein producing capabilities, and tolerance to various industry-relevant stresses. Since the secretion profiles of heterologous proteins are relatively low in industrial and laboratory strains of *S. cerevisiae*, natural isolates may offer a more diverse genetic background with increased robustness to allow for improved heterologous protein secretion. In this study, the potential of natural and industrial *S. cerevisiae* strains to secrete a core cellulase enzyme complex (CBHI, CBHII, EG and BGL), encoded by genes integrated using CRISPR/Cas9 tools, was evaluated. The effect of heterologous protein production on strain robustness and overall cell viability and growth was also evaluated, to determine the influence on resulting ethanol production from cellulosic substrates. In preliminary assays our transformants demonstrated variation in BGL activity that was dependent on the regulatory promoter sequences used, and that lower-than-expected BGL activity was obtained. Therefore, an alternative BGL-encoding gene was transformed into the host strains. Superior secretion capacity for the heterologous cellulases was demonstrated for natural isolates YI13_BECC and YI59_BECC in high temperature (37°C) and in the presence of acetic acid, respectively. However, it was noted that no single strain displayed the highest activity for all heterologous cellulases tested in a specific cultivation condition. This suggested that specific activity levels were dependent on the strain background and properties of the protein. Furthermore, increased tolerance to industry-relevant and secretion stresses was also noted for YI13_BECC and YI59_BECC compared to other transformants, suggesting increased robustness in addition to their superior secretory capacities. However, growth analyses of these superior secretors revealed reduced growth compared to their untransformed counterparts, suggesting that superior heterologous protein production was performed at the expense of biomass production. Even so, it was concluded that YI13_BECC and YI59_BECC possessed improved capabilities for heterologous protein production with inherent robustness against industry-relevant stresses. For this reason, fermentation of 20 g/L Avicel was conducted in oxygen-limited conditions using YI13_BECC and YI59_BECC without the addition of exogenous enzymes. This yielded ethanol concentrations of 4-4.5 g/L (35-40% theoretical maximum ethanol yield) after 120 hours. We conclude that YI13_BECC and YI59_BECC displayed potential to be utilised as chassis organisms on a CBP platform for bioethanol production.

CHAPTER 1: LITERATURE REVIEW

Bioethanol generated through the conversion of biological materials, such as lignocellulose, have gained much attention over the past two decades (Fatma *et al.*, 2018), as it offers a sustainable means for fuel security without threatening global food security (Kitagawa *et al.*, 2011). However, to use these lignocellulosic biomass feedstocks in industrial processes, several drawbacks need to be addressed to introduce a sustainable and cost-efficient production process. These drawbacks include: (1) costs incurred by pre-treatment of lignocellulose, (2) availability of microorganism(s) with both cellulolytic activity and ethanol-producing capabilities, (3) reduction and/or elimination of exogenous cellulase cocktails, and (4) operational costs incurred by multiple reaction units during the production process. This chapter will therefore delph into several aspects of how the lignocellulosic bioethanol production process may progress towards being sustainable and cost-efficient, with the use of microorganisms.

1.1. Bioenergy

Bioenergy can be defined as energy generated from biomass feedstocks with the goal to promote a "greener" economy (Scarlat *et al.*, 2015). For many decades, fossil fuels (i.e., coal, natural gas, and crude oil) have been used as energy sources for powering vehicles and generating electricity. However, due to their finite nature and their negative contribution to global climate change, the demand for renewable energy sources became imperative (Bryant and Love, 2017). By generating energy from biological sources and attaining an almost closed-carbon cycle, significant reductions in greenhouse gas (GHG) emissions can be achieved, hence promoting low carbon economic growth aimed at protecting ecosystems (Figure 1.1) (Timonen *et al.*, 2021).

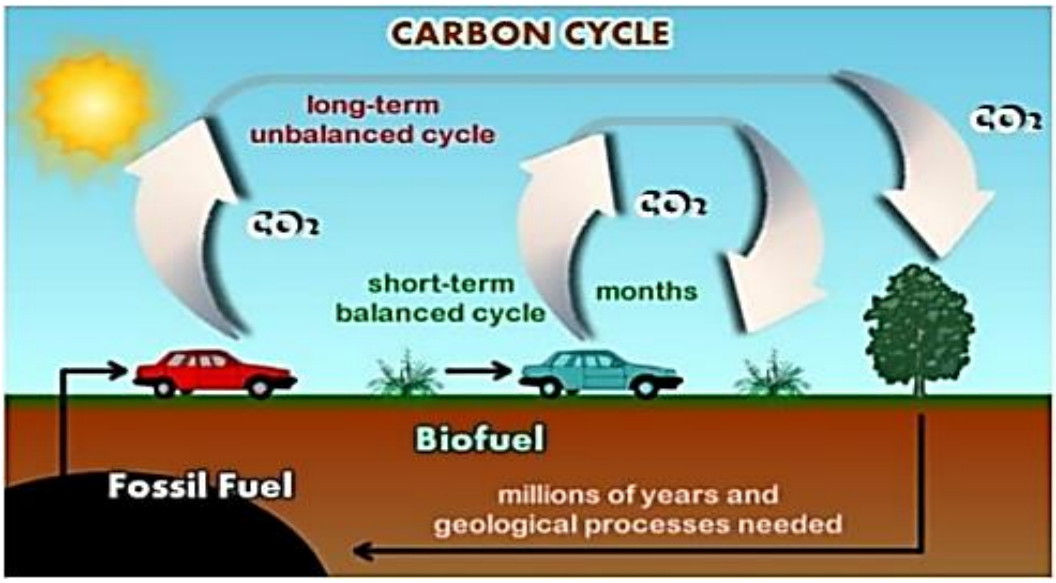

Figure 1.1: Carbon cycle for fossil fuel and/or bioethanol when used as fuel source for transportation. Fossil fuels have a long-term unbalanced carbon cycle as geological processes requires millennia to renew fossil fuel resources. In comparison, biofuels have a short-term balanced cycle, i.e., carbon dioxide released in the environment is consumed by plants for subsequent use as feedstocks for biofuel production (Suh, 2014).

Biomass-derived energy sources, alternatively known as "biofuels", offer several advantages including renewability, biodegradability, environmental feasibility, and energy security (Callegari *et al.*, 2020; Oh and Jin, 2020; Passoth and Sandgren, 2019). An example is bioethanol, the most prominent liquid biofuel produced globally for use in transportation and several industrial applications. According to the World Bioenergy Association 2020 report (www.worldbioenergy.org), 160 billion litres of biofuels were produced globally in 2018, of which bioethanol contributed a share of 62%, followed by biodiesel and other biofuels (renewable biodiesel, cellulosic bioethanol, etc.) at 26 and 12%, respectively. The 2019 IEA report (www.iea.org) forecasted a 24% increase for renewable energy in the form of biofuels by 2024. Based on these statistical datasets, a steady growth rate in biofuel production would significantly boost energy security globally, hence relieving dependence on fossil fuel-generated energy sources. However, despite these positive projections for global biofuel growth, a limitation in the availability of suitable feedstocks and the cost of production remains as significant barriers in its sustainability.

1.2. Bioethanol

Bioethanol refers to ethanol produced from biological feedstocks (agricultural products etc.) for use as substitute and/or supplement for conventional gasoline, or for use within industrial applications (Alam *et al.*, 2019). The properties of bioethanol that makes it advantageous for

use as a substitute for fossil fuel are as follows: (1) it can be obtained from renewable sources; (2) has a high oxygen (O_2) content for cleaner combustion so as to reduce toxic GHG emissions; (3) a high octane number for increased performance; (4) high heat of vaporisation to enhance the volumetric efficiency of gasoline-ethanol blends; and (5) the by-products formed as a result of incomplete combustion have lower toxicity than those formed from other alcohol fuels (Halder *et al.*, 2019; Susmozas *et al.*, 2020). In addition, utilising bioethanol would significantly promote growth in the local economy and ensure improved health conditions. However, although the utilisation of such a cleaner fuel would allow for maintaining an equilibrium in the global energy balance, the availability of feedstocks for its production remains an obstacle.

1.2.1. 1G and 2G bioethanol feedstocks

Bioethanol is classified according to various manufacturing categories based on the type of biomass used as feedstock (Susmozas *et al.*, 2020). Edible food crops obtained from the agricultural industry that are rich in starch or sucrose are used to produce first-generation (1G) bioethanol (Figure 1.2), ensuring large amounts of fermentable sugars are available for hydrolysis by fermentative microorganisms (Bhatia *et al.*, 2017).

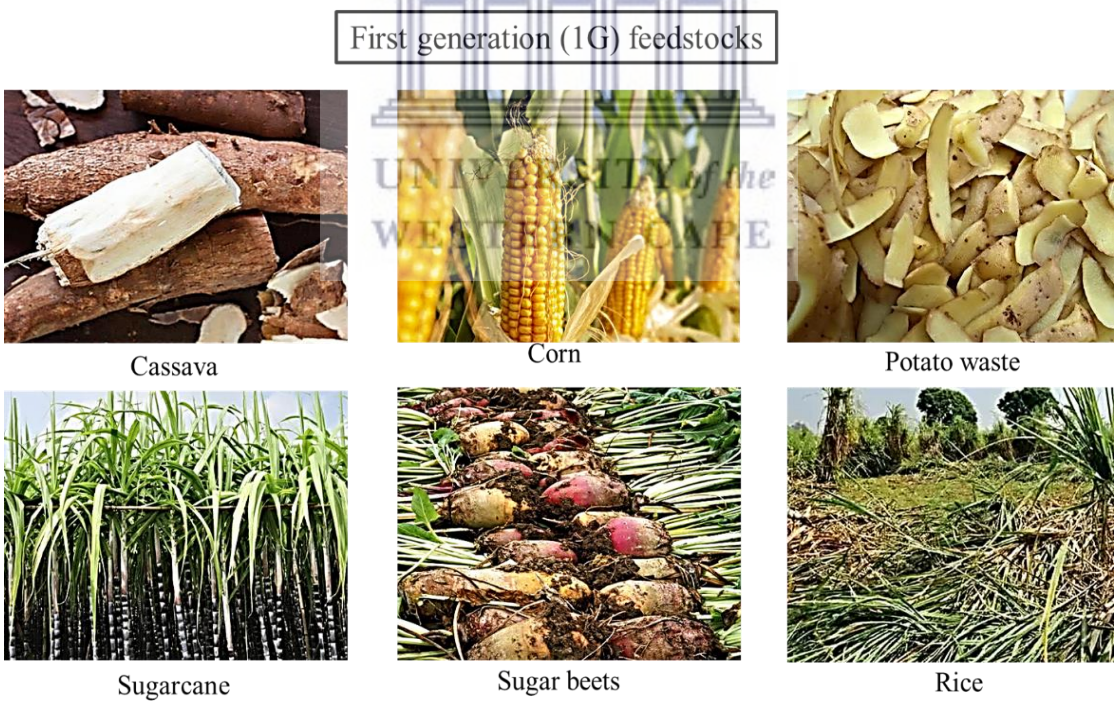

Figure 1.2: Feedstocks used to produce first-generation (1G) bioethanol. Crops rich in sucrose (sugar beets and sugarcane) and starch (cassava, corn, potato waste, and rice) are subjected to a series of process steps to yield sucrose and starch, respectively. The obtained sucrose can be directly used in fermentations; however, starch must be hydrolysed by alpha-amylase to yield glucose for the fermentation.

Globally, more than 97.8 billion litres of 1G bioethanol are produced annually, with the United States of America (USA) and Brazil being the main producers (Carpio and de Souza, 2019). As reviewed by Muktham and co-workers (2016), high yields of feedstocks for 1G bioethanol production is largely dependent on the location and dominant agricultural product in a given region. Thus, to produce large quantities of 1G bioethanol at a sustainable rate, the availability of large quantities of these food crops at reasonably low prices is required. However, due to rapid population growth, utilisation of such large quantities has raised ethical concerns regarding food security, land and water usage changes, as well as destruction of natural ecosystems (Oh and Jin, 2020; Passoth and Sandgren, 2019). To mitigate these concerns, research efforts have instead targeted non-edible agricultural and forestry residues, containing high lignocellulosic content, such as wood, straw, grasses, municipal and agricultural wastes, and dedicated food crops, for bioethanol production (Figure 1.3) (Azhar *et al.*, 2017; Nigam and Singh, 2011; Saini *et al.*, 2015).

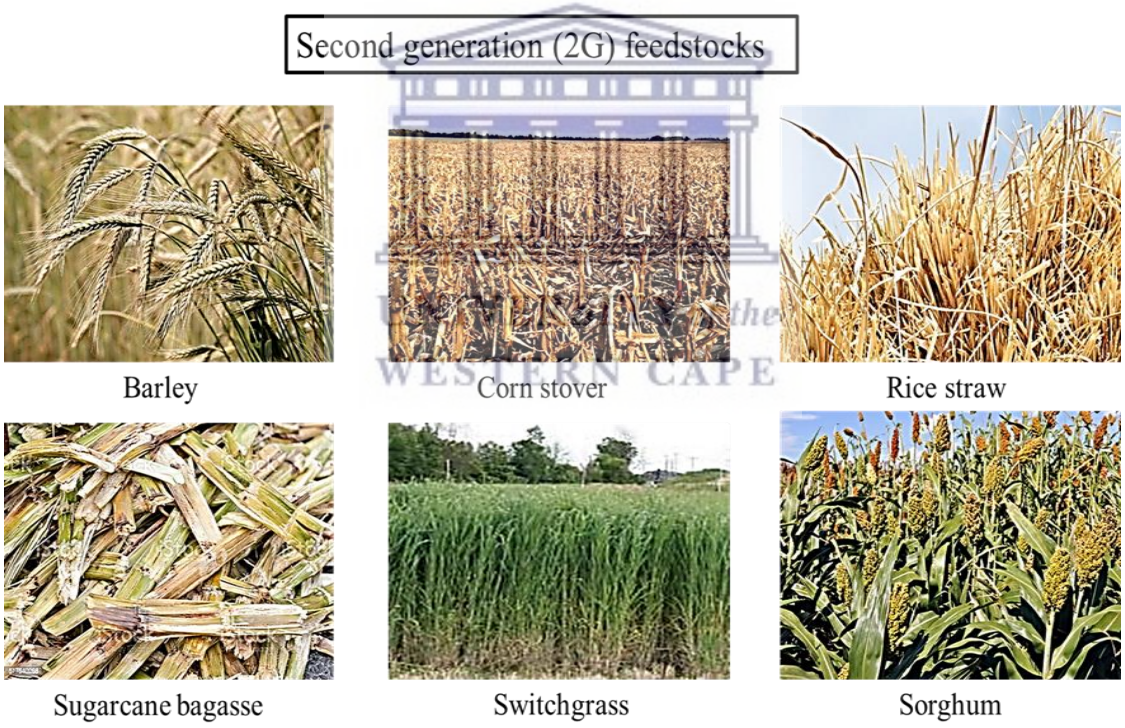

Figure 1.3: Feedstocks used to produce second generation (2G) bioethanol. Non-edible plant residues contain high lignocellulosic content (cellulose, hemicellulose) that can be used as feedstocks in microbial fermentations. The polysaccharides are hydrolysed with the aid of hydrolytic enzymes to yield five and six-carbon sugars, that are used by fermentative microorganisms as carbon sources.

Lignocellulose is the most abundant organic biomass on Earth, with an estimated production of about 181.5 billion tonnes annually (Dahmen *et al.*, 2018; Morales *et al.*, 2021). This organic matter constitutes the major structural component of woody and non-woody plants, and its constituents have chemical properties of great biotechnological value (Howard *et al.*, 2003). For bioethanol production practices, advantages of lignocellulosic biomass include: (1) wide availability in large quantities (Jansen *et al.*, 2017), (2) availability at relatively low cost (Claes *et al.*, 2020), (3) environmental benefits and (4) reduced land usage for cultivation of dedicated crops, since large quantities of biomass can be obtained from a single plant (Saini *et al.*, 2015). Although these lignocellulosic biomasses are non-edible residues, an extraction limit should be established to minimize disturbances in the carbon-nitrogen balance and nutrient availability of the soil that may affect the agricultural soil quality and subsequent biomass yields (Olguin-Maciel *et al.*, 2020). A further advantage offered by utilization of lignocellulosic biomass for fuel production is improved waste disposal management by the reduction in combustion-based practices commonly employed for its disposal. Not only does this hold benefits for the environment, but it also helps with promoting local economic growth.

1.3. Steps in cellulosic bioethanol production

1.3.1. Pre-treatment of lignocellulose

1.3.1.1. Composition and properties of lignocellulose

Lignocellulose is defined as biomass derived from the plant cell walls of shoots, leaves, and stems in living plants, wood, agricultural residues, and forest litter (Santhanam *et al.*, 2012). These biomass feedstocks consist of three major structural components, namely, cellulose (40-60%), hemicellulose (20-40%), and lignin (10-25%) (Morales *et al.*, 2021; Valenzuela-Ortega and French, 2019), as depicted in Figure 1.4. In addition, trace amounts of pectin, glycoproteins, lipids, minerals, and fixed carbon also form part of the lignocellulosic structure, all of which varies with the plant source (Althuri *et al.*, 2017; Santhanam *et al.*, 2012).

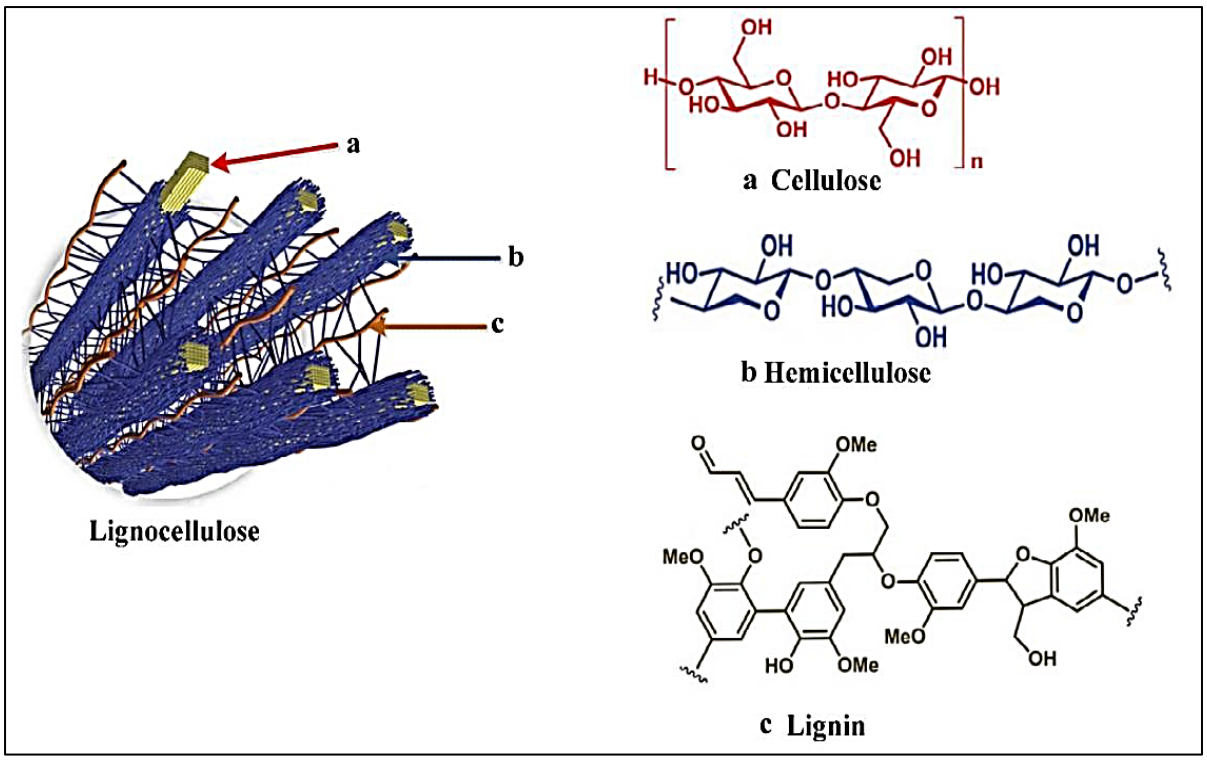

Figure 1.4: Diagram illustrating the general composition of lignocellulosic biomass. (a) Cellulose consists of long chains of β-glucose monomers that are tightly packed into fibrils via hydrogen bonds. To protect the cellulose fibrils from chemical and/or enzymatic attacks, cellulose is coated with (b) hemicellulose, which consists of short, highly branched monomers of hexose and pentose sugars. As an added protective measure, cellulose and hemicellulose are also coated with (c) lignin. This latter organic compound serves as protective layer against biotic and abiotic stresses, in addition to enabling transportation of nutrients and water through the plant's vascular system (Yusuf and Inambao, 2019).

Cellulose is a linear polymer that consists of long chains of β-glucose monomers (hexose sugar, $C_6H_{10}O_5$) linked via β-(1,4)-glycosidic bonds (Santhanam *et al.*, 2012). The number of glucose units are in the range of 2000-20000 units; however, it depends on the origin of the lignocellulosic biomass. Repeating units of β-(1,4)-glycosidic-linked glucose monomers are referred to as cellobiose, a reducing sugar. Cellulose chains contain reducing- and non-reducing ends, which provide directionality to the chain. These homopolymer chains are then tightly packed into microfibrils that are linked to one another via hydrogen bonds (Santhanam *et al.*, 2012; Yusuf and Inambao, 2019; Zoghlami and Paes, 2019). Within the fibrils, cellulose chains have highly ordered (crystalline) and low-ordered (amorphous) regions. Within the crystalline regions, cellulose chains linked via hydrogen bonds are stacked on one another via van der Waals interactions, yielding a hydrophobic macromolecule resistant to chemical and enzymatic attacks (Santhanam *et al.*, 2012). In addition, this strong inter- and intramolecular strength allows cellulose to be relatively insoluble in standard solvents, like water (Soltanian *et al.*, 2020). Amorphous regions, in turn, are less ordered structures highly amenable to chemical

and enzymatic hydrolysis, hence these regions are more easily degraded to release free chain ends and glucose monomers for use by fermentative microorganisms.

To protect cellulose microfibrils and to lend structural rigidity and hydrophobicity to the overall plant cell wall, cellulose is coated with hemicellulose and polyphenolic lignin (Valenzuela-Ortega and French, 2019). Hemicellulose consists of various monosaccharide subunits arranged as short, highly branched polymers of pentose (D-xylose - $C_5H_8O_4$) and hexose (D-glucose and D-mannose) sugars, yielding an amorphous structure that is more readily hydrolysed than cellulose (Yusuf and Inambao, 2019). Compared to pectin contained in the hemicellulose and cellulose envelope, hemicellulose also tends to be more hygroscopic, less acidic, and hydrophilic (Santhanam *et al.*, 2012). Lignin $[C_9H_{10}O_3(OCH_3)_{0.9\text{-}1.7}]_n$, in turn, is an organic compound consisting of three different phenolic monomers joined together via a set of linkages to form a matrix with various functional groups (Yusuf and Inambao, 2019). This organic compound is bound to hemicellulose via cinnamic acid ester linkages and function by providing stiffness and strength to the plant cell wall. Furthermore, lignin enables transportation of water and nutrients through the plants' vascular system and provide protection against biotic and abiotic plant stresses (Santhanam *et al.*, 2012). Although these polymers provide structural properties and excellent protection to plant cells, the recalcitrant and complex structure of the polymers poses difficulty for saccharolytic enzymes to access the polysaccharides for the release of sugar monomers used by fermenting microorganisms in the bioethanol production process (Valenzuela-Ortega and French, 2019; Zoghlami and Paës, 2019). Therefore, feedstocks are subjected to pre-treatment processes to remove lignin structures prior to saccharification, increasing the efficiency of saccharification and downstream bioethanol production (Soltanian *et al.*, 2020).

1.3.1.2. Pre-treatment and processing technologies

Pre-treatment of lignocellulosic biomass is directed at destabilising the rigid plant cell wall (Valenzuela-Ortega and French, 2019; Wang *et al.*, 2020), to enhance accessibility and biodegradability of cellulose and hemicellulose structures (Soltanian *et al.*, 2020). An ideal pre-treatment process strives to achieve various objectives, namely, (1) to increase the surface area for improved accessibility to cellulose by saccharolytic enzymes; (2) to degrade lignin-hemicellulose linkages; (3) to decrease the crystallinity of cellulose; (4) to increase the solubility of hemicellulose and lignin; (5) to promote enzyme digestibility; (6) to diminish sugar loss; (7) to reduce the energy demand during downstream processes; and, (8) to minimise

the formation of inhibitory compounds that may affect the functionality of fermenting microorganisms (Cheah *et al.*, 2020; Soltanian *et al.*, 2020). However, due to the complex structure and huge variation among different lignocellulosic feedstocks, pre-treatment processes are selected based on the type and properties of a particular biomass. The pre-treatment processes commonly used includes physical, chemical, physicochemical, biological, or a combination of different pre-treatment methods (Fatma *et al.*, 2018), as illustrated in Figure 1.5.

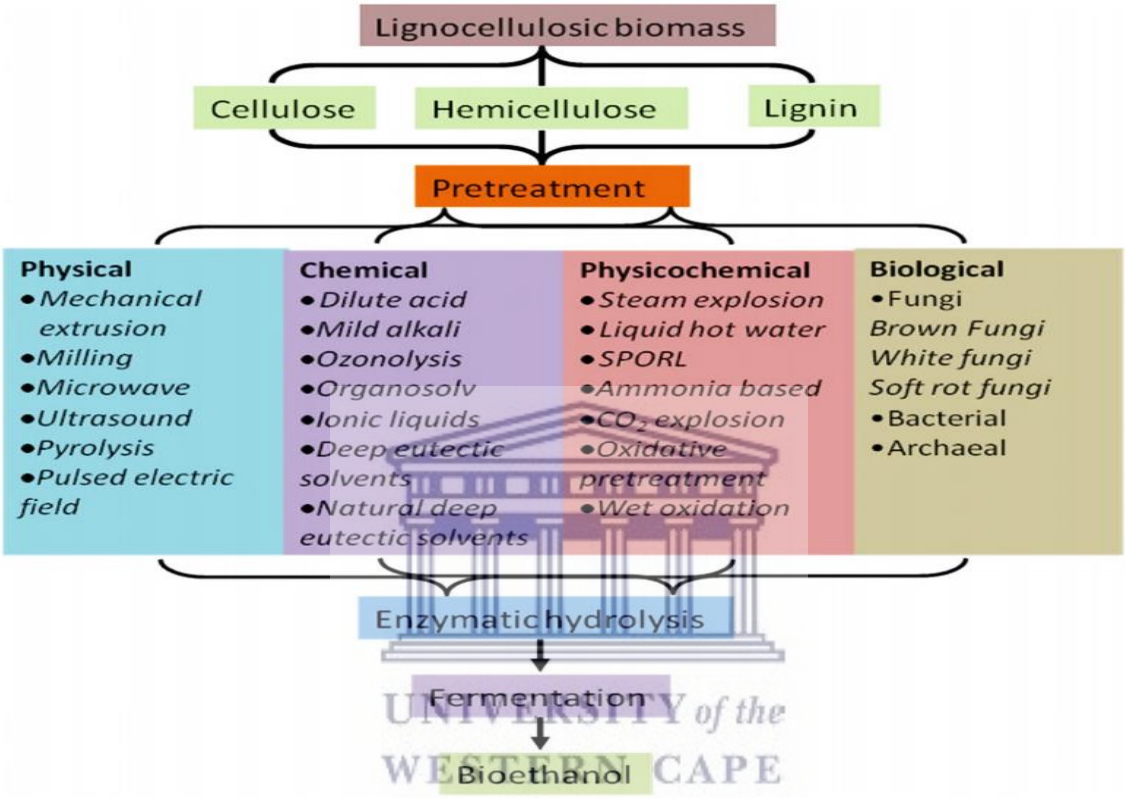

Figure 1.5: Pre-treatment processes used for lignocellulose destabilisation. Physical, chemical, biological, physico-chemical, or a combination of these processes can be used to destabilise rigid plant cell walls to increase the accessibility of cellulolytic enzymes to cellulose structures for the release of fermentable glucose monomers (Kumar and Sharma, 2017).

Although these pre-treatment processes allow for sufficient destabilisation of various lignocellulosic materials, each pre-treatment method has its own advantages and disadvantages (Sharma *et al.*, 2019). For instance, chemical and physico-chemical pre-treatment methods are the most preferred methods on a commercial scale. These methods allow for sufficient increase in the surface area of lignocellulosic biomass to enhance the accessibility of saccharolytic enzymes in the downstream saccharification process. However, both methods cause formation of inhibitory components in pre-treated materials, and the energy requirement and overall cost

of the processes are relatively high. Conversely, physical and biological pre-treatments often do not involve the introduction of inhibitory components that may have an adverse effect on microorganisms or enzyme functionality in downstream processes, but the energy, time and cost associated with these pre-treatment methods do not fit within industrial requirements (Cheah *et al.,* 2020). Overall, pre-treatment of lignocellulose is estimated to contribute up to 40% to the total cost of the entire bioethanol production process (Branco *et al.,* 2019). Furthermore, detoxification of pre-treated materials to remove inhibitory components prior to saccharification adds further costs to the production process.

1.3.1.3. Inhibitory components

The growth and functionality of microorganisms used in bioconversion processes are affected by several conditions within the production setting (Branco *et al.,* 2019). In general, physicochemical conditions such as temperature, pH, osmolarity and the composition of the growth media, play a pivotal role in the success of fermenting microorganisms to convert feedstocks into end-products. An imbalance in any of these conditions could severely impair cellular growth and metabolic processes which may lead to the impairment of the entire fermentation process. Of particular importance to the success of 2G bioethanol production is the microorganism's ability to exhibit robustness against inhibitory components, such as sugar-derived aldehydes, short-chain organic acids, and aromatic compounds (Figure 1.6) present in the pre-treated hydrolysate, to ensure that maximum yields and titers of end-products are achieved (Olguin-Maciel *et al.,* 2020, Sjulander and Kikas, 2020).

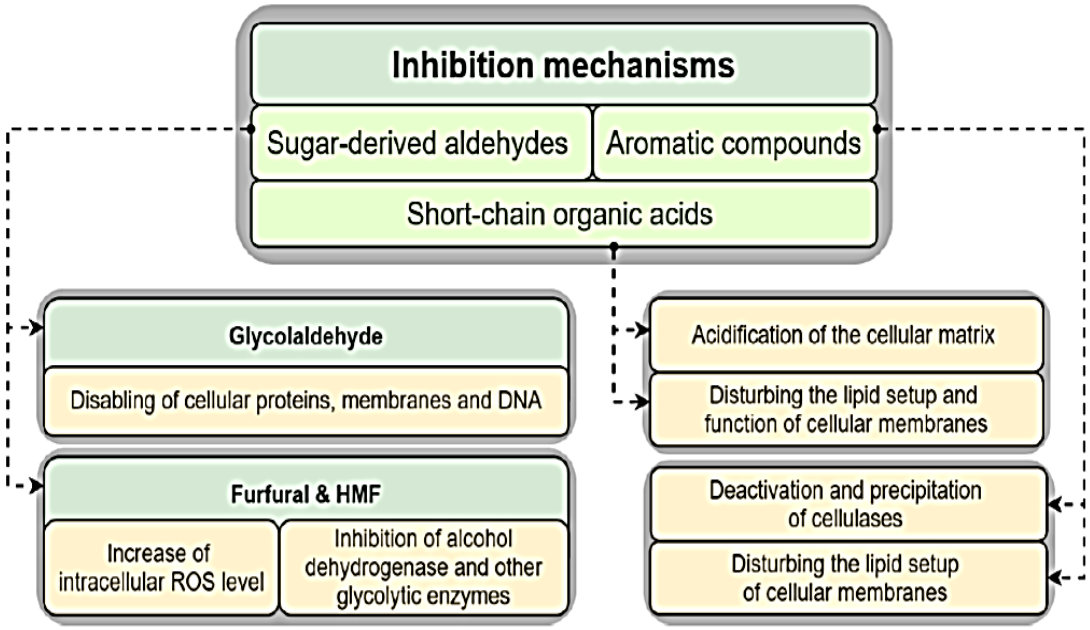

Figure 1.6: Inhibition mechanisms of pre-treatment derived inhibitors on yeast cellular growth. Inhibitors found in pre-treated lignocellulosic hydrolysates pose barriers for efficient functioning of microorganisms, as these compounds have the ability to severely impair cellular growth and metabolic processes. HMF, hydroxymethylfurfural (Sjulander and Kikas, 2020).

During pre-treatment of lignocellulosic materials, dehydration of hexose and pentose sugar polymers yield inhibitory components, such as glycolaldehyde, furfural and hydroxymethylfurfural (HMF) (Sjulander and Kikas, 2020). Glycolaldehyde, a by-product of hexose and pentose sugar dehydration, can alter cellular mechanisms through the formation of covalent linkages to proteins, DNA, and amino residues contained in cell membranes. This disturbance has a negative effect on cell viability and reproduction, leading to cessation in cell biomass production. Furfural and HMF, by-products of pentose and hexose sugar dehydration, respectively, in turn inhibit the enzymes involved in glycolysis, leading to impaired glycolysis and ultimately stuck fermentations. In addition, these compounds also induce the accumulation of reactive oxygen species (ROS) in cells, causing damage to several macromolecules (Deparis *et al.*, 2017; Liu *et al.*, 2020; Sjulander and Kikas, 2020). Although these components have such severe effects on the viability and survival of yeast cells, they do not exhibit any inhibitory effect on the activity of cellulolytic enzymes during lignocellulosic enzymatic hydrolysis (Sjulander and Kikas, 2020).

The degradation of lignocellulosic structural components also yields short-chain organic acids, usually in the form of weak acids (Sjulander and Kikas, 2020). High extracellular concentrations of these acids in their undissociated forms could cause diffusion across cell

membranes, resulting in an intracellular acidification that has several negative effects for yeast cells, namely: (1) a decrease in the synthesis rate of DNA and RNA molecules within cells, (2) impairment of nutrient uptake, (3) disruption of cell membrane functions, and (4) effects on lipid metabolism. However, despite their negative effects they may help in the improvement of fermentations, and similarly to furfural and HMF, they do not exhibit any inhibitory effect on cellulolytic enzymes during enzymatic hydrolysis. Examples of such weak acids include acetic acid and formic acid.

Due to the huge impact pre-treatment inhibitors has on fermenting microorganisms, several methods have been proposed to detoxify pre-treated lignocellulosic hydrolysates prior to the actual fermentation process (Klosowski and Mikulski, 2021). For instance, the optimisation of biomass pre-treatment methods has been proposed and would involve a limitation in the utilisation of factors that would aid in increased inhibitory component concentrations. However, if certain chemicals or process parameters (e.g., temperature) were to be optimised during pre-treatment, it may result in ineffective pre-treatment or an even higher demand for downstream energy and process costs. Thus, the more widely used approach currently is the utilisation of microorganisms with increased tolerance to these toxic inhibitors. Increased inhibitor tolerance in microorganisms can be achieved through several methods, namely, by (1) overexpressing genes involved in specific cellular processes, (2) overexpressing and manipulating single/multiple genes involved in stress response, (3) adapting microbial strains to harsh environments over time, or (4) using microbial strains naturally robust to harsh environmental conditions. Utilisation of these biological systems allows neutrality in the energy balance and a significant decrease in production costs, hence ensuring a more sustainable production process (Caspeta *et al.,* 2015).

1.3.2. Enzyme production by fermentative microorganisms

The production of microbial enzymes and proteins has gained widespread recognition across several industries, due to environmental safety, economical value, and a means to reduce costs associated with the use of chemicals (Singh *et al.,* 2016). Several microbial enzymes are produced on a commercial scale for use in industries related to food, animal feed, beer and wine, waste management, pharmaceutical applications, and biofuels (Behera *et al.,* 2017; Nigam, 2013). For instance, proteases, lipases, and amylases are widely employed in the food, pharmaceutical, animal feed and detergent industries for the breakdown of polymers into simpler compounds. In addition, laccases are commonly used in bioremediation processes for

treatment of hazardous and recalcitrant chemicals present in industrial effluents (Nigam 2013). According to Gurung and co-workers (2013), the estimated global enzyme production market in 2010 reached $3.3 billion, with a forecasted increase to $4 billion in 2015. Although these estimates include the entire enzyme production market, microbial cellulases were estimated to contribute approximately 20% to the technological enzyme sector, thereby making it the third-largest produced enzyme catalyst worldwide (Bhati *et al.,* 2020). Due to increase demand for sustainable production of bioethanol, these projections are set to increase gradually over the next few years.

1.3.2.1. Microbial cellulases: availability for industrial applications

Cellulases are a group of enzymes belonging to the glycoside hydrolase family (GH) (EC.3.2.1.-) that are expressed by a broad spectrum of actinomycetes, bacterial and fungal organisms (Diaz-Rincon *et al.,* 2017) when cultivated on cellulosic materials (Kuhad *et al.,* 2011). The expression and secretion of fungal cellulases is simpler than the cellulosomes produced by some bacteria, hence fungal cellulase producers are often used in industry as cell factories for commercial enzyme production, or as donors of genes for heterologous enzyme production in other cell factories (Kuhad *et al.,* 2011; Zhao *et al.,* 2018). Among the most extensively studied fungal cellulase producers, *Trichoderma reesei* is of great importance as it expresses the core complex of enzymes necessary for the complete hydrolysis of cellulosic materials (Diaz-Rincon *et al.,* 2017). Other major fungal cellulase producers include *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., *Cladosporium* sp., and *Humicola* sp. (Diaz-Rincon *et al.,* 2017; Leo *et al.,* 2019). Due to the complex cellulase systems present in most bacterial cellulase producers, the most exploited producers are *Bacillus* sp., *Clostridium* sp., and *Pseudomonas* sp. (Sukumaran *et al.,* 2005).

Microbial cellulases have been employed for several decades in various industries as hydrolytic and/or depolymerising enzymes (Behera *et al.,* 2017; Kuhad *et al.,* 2011; Nigam, 2013; Valenzuela-Ortega and French, 2019). These industries include animal feed, detergent, textile, paper and pulp, wine and brewing, and food and dairy (Leo *et al.,* 2019), as shown in Figure 1.7. However, due to the increasing demand for the sustainable production of biofuels, particularly bioethanol from cellulosic materials, the utilisation of cellulases on a commercial scale has skyrocketed (Juturu and Wu, 2014).

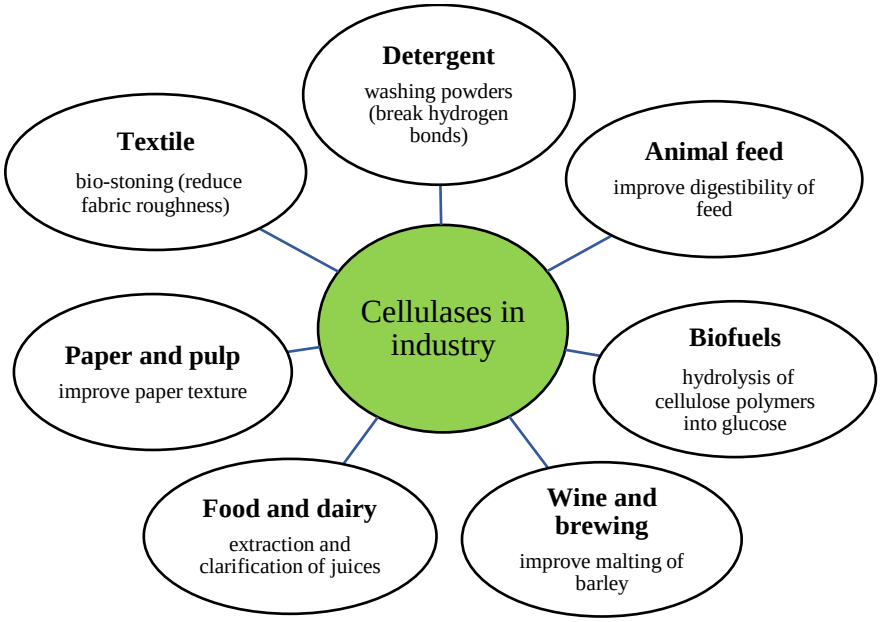

Figure 1.7: Industrial applications of cellulases. Cellulases are commonly employed as hydrolytic and/or depolymerizing enzymes for processing of food commodities and in the paper and pulp, detergent, and textile industries.

The optimal functionality of enzymes in each respective industrial application is greatly dependant on the native organism and the environment from which the organism was isolated (Ejaz *et al.,* 2021). For example, cellulolytic organisms isolated from extreme temperature environments like hot springs have the potential to yield cellulases that are thermostable. As reviewed by Juturu and Wu (2014), a *Chrysosporium lucknowense* strain C-1 was isolated from alkaline soil, hence the strain expressed multiple cellulases that were active in neutral and alkaline pH conditions. These enzyme preparations could therefore be employed in textile washing applications, which yielded superior activity to current commercial enzyme preparations. Additionally, a *T. reesei* F-418 strain cultivated on alkali pre-treated rice straw at a pH of 4.8 yielded cellulase yields of 16.2 $IU.g^{-1}$ after 5 days. This strain was able to grow over a narrow pH range, hence yielding cellulases with similar characteristics. Therefore, the industrial application in which cellulases are required selects enzymes based on the environmental, functional, and structural properties of both application and enzymes.

1.3.2.2. Structure and organisation of cellulolytic enzymes

The complex cellulase enzymes responsible for the cleavage of β-1,4-glycosidic linkages present in cellulose and hemicellulose structures are classified as glycoside hydrolases (GHs) (Cragg *et al.,* 2015). These enzymes are documented in an exclusive database called carbohydrate-active enzymes (CAZymes) (http://www.cazy.org) based on their catalytic and

carbohydrate-binding properties (Leo *et al.*, 2019). Within the database, enzymes are classified into different structural families with a variety of related activities (Valenzuela-Ortega and French, 2019). Although all cellulases exhibit specificity towards β-1,4-glycosidic linkages, they differ based on their specificities for crystallinity and degree of polymerisation (DP) of their cellulose substrate (Santhanam *et al.*, 2012).

GHs employ general acid-base chemistry to initiate hydrolysis of glycosidic linkages in cellulose structures (Medie *et al.*, 2012; Rabinovich *et al.*, 2002). The structural architecture of GHs is based on the presence of one or more catalytic domain (CD) and/or carbohydrate binding module (CBM) (Hildén and Johansson, 2004; Santhanam *et al.*, 2012). In the catalytic domain, an active site is located where the hydrolysis of glycosidic linkages in cellulose is initiated. The topological arrangement of a GHs' active site allows for its classification according to three different types, namely, (1) tunnel shaped for processive exo-attacks, (2) cleft shaped for endo-attacks, and (3) crater/pocket shaped for substrate degradation by attacking at the chain ends, as shown in Figure 1.8 (Thapa *et al.*, 2020). In the active site, hydrolysis is initiated via two stereo-chemically different mechanisms, namely, inverting and retaining, where the former mechanism generates cellobiose as an α-anomer, and the latter mechanism carries out trans-glycosylation to yield cellobiose as β-anomers (Medie *et al.*, 2012; Santhanam *et al.*, 2012).

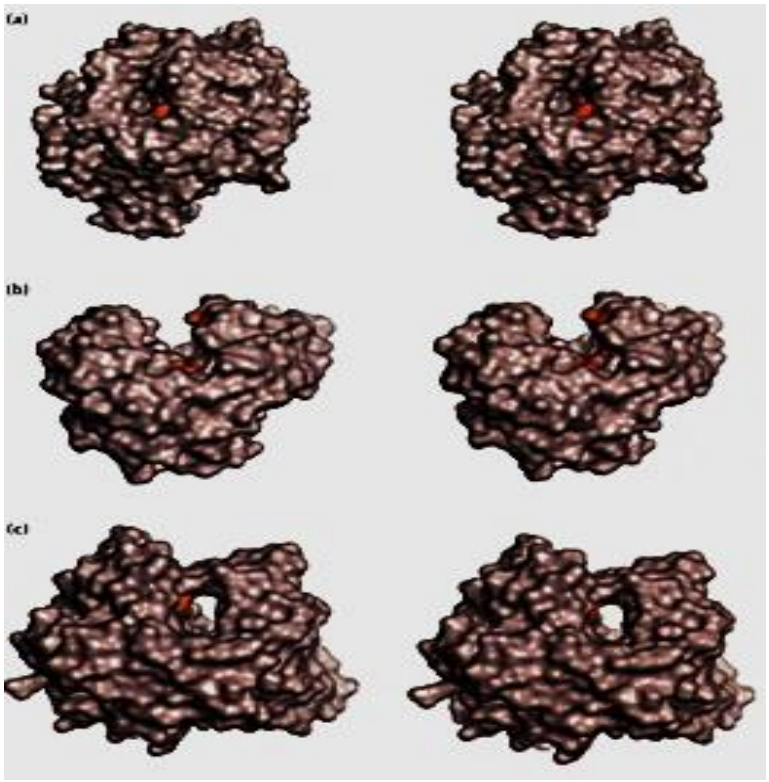

Figure 1.8: Glycoside hydrolase (GH) active site topologies. (a) Crater/pocket shaped (glucoamylase found in *Aspergillus awamori*); (b) Cleft (endoglucanase E2 found in *Trichoderma fusca*); and (c) Tunnel shaped (exo-glucanase II found in *Trichoderma reesei*). Red areas signify proposed catalytic sites (Davies and Henrissat, 1995).

To allow for the targeting of CDs to cellulose substrates, cellulases are usually equipped with CBMs at either the C-or N-terminal end of the CD (Ilmén *et al.,* 2011; Valenzuela-Ortega and French, 2019). Although CBMs display no detectable hydrolytic activity, they aid in the enhancement of CD activity by binding to the surface of cellulose fibrils, hence bringing the CD in closer proximity to immobile substrates. The CBMs of many GHs vary in size from 4-20 kDa containing approximately 33-36 amino acid residues (Hildén and Johansson, 2004; Santhanam *et al.,* 2012). In fungal organisms, CBMs are linked to the CD via a highly glycosylated flexible linker peptide (shown in Figure 1.9), which allows these modules/domains to function independently whilst keeping them together (Juturu and Wu, 2014). Hydrophobic interactions generated by the organisation of β-sheets in the CBM allows for an increase in the binding stability of the module to the immobile substrate.

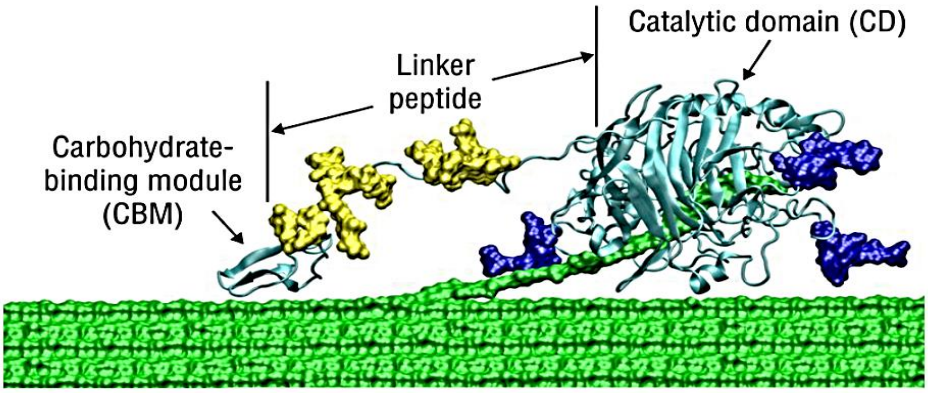

Figure 1.9: Catalytic domain (CD) attached to carbohydrate binding module (CBM) via a linker peptide (Barcelos *et al.,* 2015).

The expression of cellulases in bacterial and fungal systems are generally perceived as complexed and non-complexed, respectively (shown in Figure 1.10) (Juturu and Wu, 2014). In the complexed bacterial system, cellulases are clustered into cellulosomes on the bacterial cell wall. In these cellulosome structures, cellulase subunits are positioned on a scaffolding protein which consists of cohesins and dockerins (Ejaz *et al.,* 2021). Conversely, non-complexed fungal systems consist of free and/or cell-surface attached cellulases, where the cellulase units contains CD(s) and CBM(s) linked via a linker peptide.

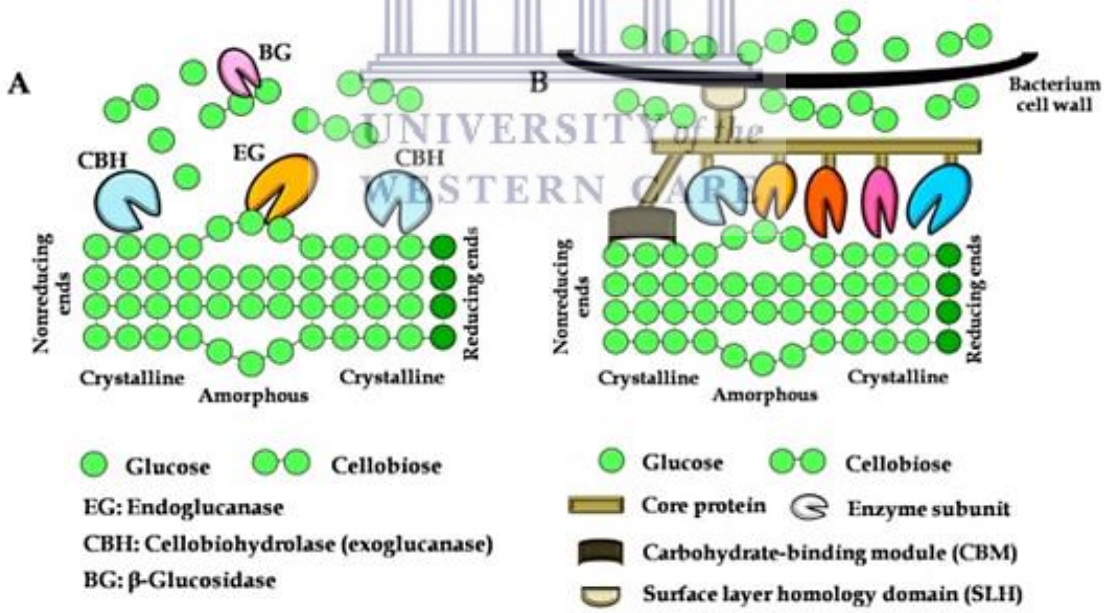

Figure 1.10: Cellulolytic enzyme systems in (A) fungi and (B) cellulosome-producing bacteria. In fungal systems, cellulases are secreted as free enzymes, where individual enzymes act in synergy to cleave cellulose into glucose monomers for utilization by fermentative microorganisms. In bacteria producing cellulases in cellulosomes, individual cellulases are anchored on a scaffold attached to the bacterial cell wall, whilst still acting in synergy to yield glucose monomers for utilization by the microorganism (Madadi *et al*., 2017).

Heterologous cellulase production often uses cellulase-encoding genes from filamentous fungi, due to their particularly high expression levels in the native hosts (Chang *et al.*, 2013). To date, cellulases expressed by superior fungal producers, such as *Trichoderma* sp., and *Aspergillus* sp., have had their structural and functional properties elucidated to a great extent, making it possible to re-produce these enzymes in heterologous hosts. Unlike fungi, the complexity of the bacterial cellulosome architecture makes expression in eukaryotic hosts more difficult, due to the post-translational modifications initiated by these cell lines, which results in low cellulase expression levels and less stable complexes. Nevertheless, compared to fungal cellulases, bacterial cellulases often exhibit increased thermostability, higher specific activity and can function across a broader pH range (Munjal *et al.,* 2015).

1.3.2.3. Cellulolytic enzymes required for full cellulose hydrolysis

1.3.2.3.1. Endoglucanase (EG)

Endo-β-1,4-glucanases (EC 3.2.1.4) are a class of GH produced by fungal and bacterial species, such as *Trichoderma, Bacillus, Clostridium, Paenibacillus,* and *Saccharophagus* (Thapa *et al.,* 2020). According to the CAZy database (http://www.cazy.org) endo-β-1,4-glucanases can be classified into 13 distinct families according to their catalytic activities; however, despite these differences, all these enzymes function similarly in their ability to randomly act on the internal bonds in the amorphous regions of cellulose fibrils to expose free reducing and non-reducing chain ends on individual oligosaccharide chains (Garvey *et al.,* 2013; Thapa *et al.,* 2020), each with varying DP (Barcelos *et al.,* 2015).

Several microorganisms of bacterial and fungal origin produce endoglucanase at high levels, however, endoglucanase II (Cel5A) from *T. reesei* is considered to be one of the most abundant endoglucanases with the highest catalytic activity when at pH values between 4 and 5 (Lee *et al.,* 2011; Qin *et al.,* 2008; Samanta *et al.,* 2012; Tjandra *et al.,* 2020). In addition, *T.r.*Cel5A is said to account for about 55% of endoglucanase activity in *T. reesei*, based on the total secreted endoglucanase proteins in the fungus (Lee *et al.,* 2011; Samanta *et al.,* 2012). Like most *T. reesei* cellulases, *T.r.*Cel5A contains a small CBM linked to a larger CD via a heavily *O*-glycosylated linker peptide (Lee *et al.,* 2011). To achieve internal cleavage of cellulose chains, microfibrils are bound in a shallow substrate binding groove containing a deep catalytic cleft, through which the cellulose chain is thread to gain access to the enzymes' active site. Once bound in the active site, β-1,4-glycosidic bonds in the amorphous regions of the cellulose

chain are cleaved, resulting in the subsequent release of oligosaccharide chains, which will be the substrate for cellobiohydrolases (CBHs).

1.3.2.3.2. Cellobiohydrolase (CBH*)*

Exo-glucanases (EC 3.2.1.91; cellobiohydrolases; CBH) are a class of processive cellulolytic enzymes with catalytic modules categorised into GH families 5, 6, 7, 9, 48, and 74, and are found in several fungal and bacterial species (Annamalai *et al.*, 2016; http://cazy.org/). For the hydrolysis of highly ordered regions in oligosaccharide chains generated by endo-attacks, CBHs play an important role in attacking β-1,4-glycosidic bonds to generate oligosaccharide units, mainly as cellobiose, and smaller amounts of cellotriose and glucose.

CBHs secreted by filamentous cellulolytic fungi are classified into two major GH families, namely, GH6 (also referred to as CBHII) and GH7 (also referred to as CBHI) (Ilmén *et al.*, 2011), with GH7 enzymes being referred to as the most potent CBHs (Cragg *et al.*, 2015). Both CBHs share similar topological arrangement in their CD and CBM units, with slight variations. For instance, the active site of both CBHs have a tunnel-shaped conformation, through which a cellulose chain can thread to be hydrolysed by the processive action of the CBHs (Taylor *et al.*, 2018); however, their CBMs are attached at either the C- or N-terminus, for CBHI and CBHII, respectively (Ilmén *et al.*, 2011). These alternating CBM arrangements allows CBHI and CBHII to act collectively on one chain from the reducing and non-reducing chain ends, respectively (Annamalai *et al.*, 2016; Santhanam *et al.*, 2012).

1.3.2.3.3. β-glucosidase (BGL)

β-glucosidase (EC 3.2.1.21; cellobiase; BGL) is a cellulolytic enzyme that catalyses the hydrolysis of cellobiose and some cello-oligosaccharides from the non-reducing ends to liberate glucose monomers, in the final step of cellulose degradation (Mohsin *et al.*, 2019). Similar to EGs and CBHs, BGL is found in a wide range of species, and are categorised into several GH families, namely 1, 3, 5, 9, 30, and 116 (Baba *et al.*, 2015; Mohsin *et al.*, 2019; Suzuki *et al.*, 2013; www.cazy.org). Most fungal BGLs are classified into the family GH3, with the most potent BGL present in *A. aculeatus* (Baba *et al.*, 2015; Suzuki *et al.*, 2013). The potency of *A.a.BGLI* is reported to not only be towards soluble cello-oligosaccharides, but also towards insoluble cello-oligosaccharides.

Unlike EGs and CBHs, BGLs do not have CBMs, and can be extracellularly secreted or be membrane-associated in fungi (Santhanam *et al.*, 2012). The active site of GH1 BGL enzymes

are in a folded $(\alpha/\beta)_8$-barrel that contains the active site and that employs Glu for catalytic nucleophilic attacks (Molina *et al.*, 2016). GH3 BGL enzymes, on the other hand, contain their active site between a two-domain structure, consisting of $(\alpha/\beta)_8$-barrel and a $(\alpha/\beta)_6$-sandwich domains. These latter enzymes employ Asp residues for nucleophilic attacks of their substrates.

1.3.2.4. Enzymatic hydrolysis: the synergism concept

Synergism can be defined as the effect that arises when more than one entity, factor, agent, or substance act together, to yield a combined effect much greater than the sum of individual effects (Deraz, 2017). This statement holds true in cellulolytic hydrolysis, as the collaborative action of the entire cellulase complex enzymes significantly aids hydrolysis of lignocellulosic materials, by minimising inhibition of the enzymes by their respective products (Santhanam *et al.,* 2012).

For efficient cellulolytic hydrolysis to occur, a high degree of synergy should exist between endo- and exo-cellulases (EGs and CBHs) (Behera *et al.,* 2017). This synergy allows CBHs to readily act on the cello-oligosaccharide chains released by the EGs, to minimise and/or eliminate potential enzyme inhibition which may result in a halt of hydrolysis. In addition, the collaborative action of EGs with CBHs allows for an enhancement in the hydrolytic efficiency of CBHs, as CBHs are the major enzymes involved in crystalline cellulose hydrolysis. Although this synergy greatly benefits cellulolytic hydrolysis, build-up of cellobiose units by CBH action could cause inhibition of the enzyme. This inhibition could occur via two mechanisms, namely (1) cellobiose competing with the cellulose chain for binding to the active site of the enzyme, or (2) non-competitive inhibition of the enzyme's processivity by lingering in the product-binding site (Atreya *et al.,* 2016). Therefore, to ensure a limitation in CBH inhibition, the presence of a potent BGL is required for cleavage of external cellobiose units to yield glucose monomers. Furthermore, to ensure an overall efficient cellulose degradation, the expression ratios of each individual cellulolytic enzyme and the combined synergistic effect plays a pivotal role in the duration and specific rate at which cellulose can be hydrolysed to yield fermentable sugars (Gong *et al.,* 2014).

1.4. Standard technologies for bioethanol production

Various bioprocessing technologies have been reported for the bioconversion of pre-treated lignocellulosic biomass into bioethanol (Figure 1.11) (Oh and Jin, 2020). Biological conversion of pre-treated biomass requires four steps namely (1) enzyme production, (2) saccharification

and (3) fermentation of hexose as well as (4) pentose sugars. The most mature technology employed involves each step within the production process being conducted in separate reaction vessels, in a process referred to as separate hydrolysis and fermentation (SHF). This approach allows for optimal operating conditions to be maintained for each individual process, to ensure maximum yields and titres of the final end-product are achieved. However, this approach is limited by several aspects, namely, (1) expense of using multiple reaction vessels, (2) susceptibility to contamination, (3) being time-consuming, and (4) end-product inhibition during substrate hydrolysis. Therefore, consolidation of substrate hydrolysis and hexose fermentation steps has been implemented, in a process referred to as simultaneous saccharification and fermentation (SSF). With this approach, end-product inhibition during substrate hydrolysis is eliminated, as released fermentable sugars are rapidly assimilated by fermenting microorganisms. However, a major limitation to SSF is maintaining optimal operating conditions for both cellulolytic enzymes (i.e., 45-55°C) and fermenting microorganisms (i.e., 28-37°C). Therefore, a further advancement to SSF is the consolidation of all fermentation steps, including the addition of pentose-fermenting microorganisms, however whilst still adding exogenous enzymes for substrate hydrolysis. This latter approach is referred to as simultaneous saccharification and co-fermentation (SSCF). This approach allows for a significant reduction in production costs, as less maintenance and capital costs are required when less unit operations are needed. Although each of these processes has allowed for further advancement in sustainability and productivity of bioethanol, the required exogenous enzyme cocktails still contribute up to 40% of the overall production costs (Branco *et al.*, 2019).

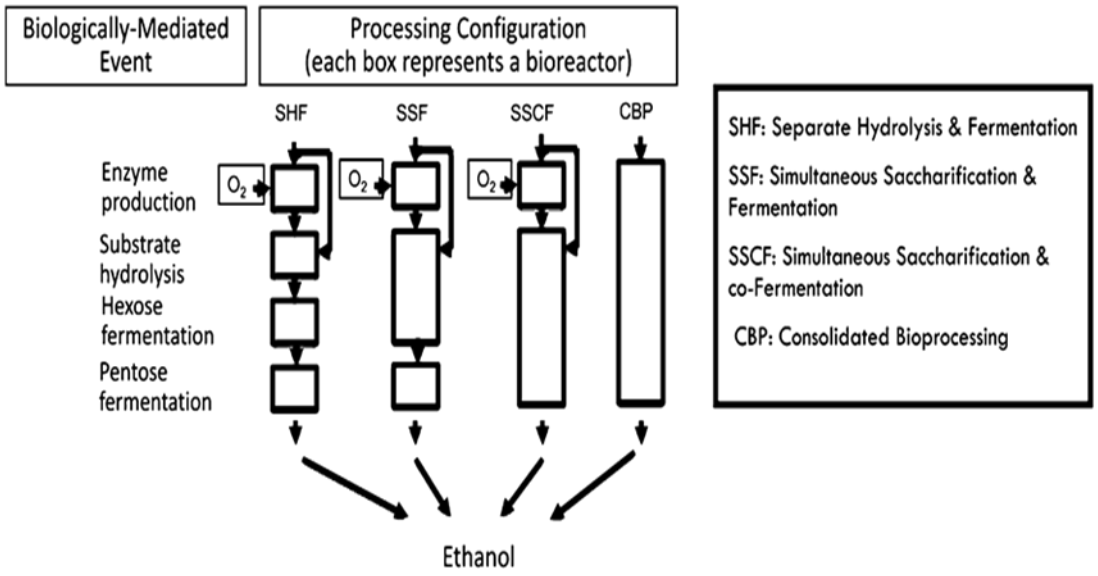

Figure 1.11: Process configurations for bioconversion of lignocellulosic biomass into bioethanol. Production of bioethanol via SHF, SSF, and SSCF requires the addition of exogenous enzyme cocktails to ensure efficient substrate hydrolysis. In the ideal CBP process, all steps in the production would be combined, without the addition of any exogenous enzymes (Saini *et al.*, 2015).

Ethanol yields and titres generated from various feedstocks vary greatly when produced using either of the beforementioned configurations (Rastogi and Shrivastava, 2017). For instance, a study conducted by Althuri and co-workers (2017) utilised a mixture of lignocellulosic substrates (i.e., annual crops and potential perennial varieties) pre-treated with a fungal laccase. To evaluate whether any enhancement in ethanol productivity could be achieved using *Saccharomyces cerevisiae*, the pre-treated lignocellulosic substrate mixture was subjected to SHF and SSF processes. The authors observed higher ethanol productivity rates for SSF (1.396 g/L/h) after 30 hours with a cellulase loading of 80 U/g, compared to the SHF which yielded ethanol productivity rates of 0.929 g/L/h after only 27.33 hours and with a higher cellulase loading (132.9 U/g). Furthermore, an increase in ethanol concentration (1.64 folds) was achieved with SSF (41.9 g/L) compared to SHF (25.4 g/L). In another study done by Pabón and co-workers (2020) rice husk pre-treated under mild conditions with sodium hydroxide was used as lignocellulosic substrate for SHF and SSF processes, with *S. cerevisiae*. Under standard operative conditions, SSF yielded the highest bioethanol yields and concentrations (38.2% and 2.17±0.03 g/L, respectively) after 72 hours compared to SHF (35.3% and 2.00±0.01 g/L, respectively) after just 12 hours. However, even though high ethanol concentrations and yields could be achieved after only 12 hours with SHF, a steady decline was observed in both bioethanol yield and concentration as the process continued, which may correspond to end-product inhibition (Oh and Jin, 2020). Conversely, Vaithanomsat and co-workers (2011)

observed that SHF and SSF yielded similar ethanol yields (21.21% and 20.67% based on pulp weight, respectively) after 72 hours, when using corn husk pre-treated with sodium hydroxide. These ethanol yields corresponded to approximately 85% of the theoretical yield. Although most studies identified SSF as the superior process compared to SHF for bioethanol production, the overall success of the production process is dependent on the type of feedstock and the pre-treatment method used.

Although these process configurations are currently being utilised in industry for bioethanol production, the cost of feedstock pre-treatment and exogenous enzyme cocktail additions presents major challenges for the sustainability and profitability of large-scale bioethanol production (Den Haan *et al.,* 2015; Kroukamp *et al.,* 2017; Oh and Jin, 2020). For these reasons, consolidated bioprocessing (CBP) is widely regarded as the best possible configuration to yield high ethanol titres at a relatively lower cost.

1.5. Consolidated bioprocessing

To advance sustainability and profitability of 2G bioethanol production, consolidation of all production steps into a single reaction vessel, using a single microorganism or consortium, without the addition of exogenous enzyme cocktails, in a process called consolidated bioprocessing (CBP) has been explored (Figure 1.11) (Kroukamp *et al.,* 2017; Valenzuela-Ortega and French, 2019). This process configuration is aimed at utilising microorganism(s) capable of (1) producing active cellulolytic enzymes necessary for hydrolysis of pre-treated feedstocks, (2) containing pathways for both pentose and hexose utilisation, and (3) tolerating toxic biomass hydrolysate environments. Implementation of this process configuration would hold several advantages for industry, namely (1) simplifying the overall process operation, (2) reducing capital investment to a minimum, (3) utilising less energy during the process, and (4) eliminating the cost of exogenous enzyme cocktail additions (Olguin-Maciel *et al.,* 2020). However, as desirable as this technology may seem, no known microorganisms with all the required CBP characteristics have been identified to date (Den Haan *et al.,* 2015). Thus, research is currently focussed on engineering organisms with certain natural traits applicable to CBP for their potential use in this process.

1.5.1. Engineering organisms for CBP

Engineering organisms for a CBP process can be achieved using one of two approaches, namely, native or recombinant (Lee *et al.,* 2017). The native strategy is based on microorganisms with the natural capacity to produce cellulolytic enzymes at relatively high

concentrations and conferring ethanol-producing capabilities to them, through engineering strategies. Conversely, the recombinant strategy involves conferring cellulolytic enzyme-producing capabilities to natural ethanol-producing microorganisms (Den Haan *et al.,* 2015; Lee *et al.,* 2017; Olguin-Maciel *et al.,* 2020; Olson et al.., 2011; Valenzuela-Ortega and French, 2019). However, regardless of the engineering approach to be followed, particular microbial attributes are imperative when selecting potential microorganisms for engineering (Adegboye *et al.,* 2021). The main attributes needed include the microorganism(s) possessing the ability to (1) attain high cell mass in a short time, (2) exhibit increased tolerance to hydrolysate inhibitors and desired end products, (3) tolerate high temperatures and low pH to minimise contamination and to improve reaction rates, (4) attain high metabolic fluxes, (5) produce individual fermentation products through fast and deregulated pathways, while (6) using a wide range of different pentose and/or hexose sugars. Although no microorganism described to date adheres to all the required attributes, metabolic and/or rational engineering approaches can be employed to engineer these strain(s).

1.5.1.1. Native approach

Microorganisms with the inherent ability to produce high titres of cellulases are often of bacterial and/or fungal origin (Bhardwaj *et al.*, 2021). For example, the *Clostridium sp.* described by Chang and Yao (2011), is a thermophilic bacterium with high cellulose decomposition capabilities in addition to ethanol production. Although this species can produce ethanol, the yields obtained were affected by additional acids produced as end-products by alternative pathways in the microorganism. To overcome the challenge of low ethanol yields in *Clostridium* sp., Liu and co-workers (2020) have explored different optimised cultivation mechanisms to achieve an increased ethanol yield for *C. thermocellum* DSM 1237. With the cultivation of the strain in the presence of 0.5% (w/v) cellobiose, an ethanol yield of 0.60 g/L with a cell biomass of 0.80 g/g was achieved. Additionally, fermentation with alkali-pretreated sugarcane bagasse resulted in an ethanol yield of 0.68 g/L, corresponding to 65.8% of the maximum theoretical ethanol yield. Lastly, upscaling the experimental conditions to a 3L fermenter trial using 8 g of alkali-treated sugarcane bagasse, with the addition of both cellulase and xylanase, a maximum ethanol yield of 0.86 g/L (83.3% theoretical yield) was achieved. These results indicate that the final yields obtained are influenced by key operational conditions in the fermentation process.

In another study conducted by Huang and co-workers (2014a) increased ethanol production yields and tolerance was evaluated in the cellulolytic filamentous fungus *T. reesei* strain CICC 40360, optimised by genome shuffling techniques. Following three rounds of genome shuffling using *T. reesei* CICC 40360, the best performing shuffled strain, HJ48, yielded an ethanol production capacity of 9.7(±0.2) g/L after 96 hours cultivation, compared to the control *T. reesei* CICC 40360 strain that yielded 2(±0.1) g/L after 120 hours cultivation. Further evaluations conducted included direct ethanol production from sugarcane bagasse, where HJ48 yielded a maximum ethanol concentration of 3.1(±0.2) g/L after 120 hours cultivation, compared to CICC 40360 which could not convert any of the sugarcane bagasse to ethanol. Although the ethanol yields had drastically decreased with the different cultivation mechanisms, genome shuffling was shown to be effective in optimising strains for improved ethanol yields.

The feasibility and sustainability of ethanol production requires ethanol titres of >40 g/L to allow for economical recovery and distillation in downstream processing (Borahona *et al.,* 2020). However, the utilisation of microorganisms engineered using these native approaches yielded ethanol titres of negligible value, and the microorganisms showed low tolerance to the ethanol concentrations in the fermentation broth. Additionally, the slow growth and difficult cultivation in fermentation media resulted in longer fermentations that were not feasible for a sustainable ethanol production process. Due to these challenges, the recombinant approach might provide better options.

1.5.1.2. Recombinant approach

Developing organisms for CBP-enabled processes using a recombinant approach requires the selection of microorganisms that can produce high ethanol titres and tolerate high ethanol and inhibitor concentrations (Olguin-Maciel *et al.,* 2020). Various microbial species have been evaluated for applicability in this approach, however, *S. cerevisiae* has been shown to be an excellent candidate due to its high ethanol production titres, process tolerance and its amenability to genetic engineering. For this reason, several studies have focussed on the engineering of this microorganism as CBP candidate and selected examples are explored in a later section.

Substantial progress has been made with the development of CBP-enabled microorganisms using the recombinant approach, however several disadvantages limit the implementation of the CBP system (Olguin-Maciel *et al.,* 2020). For instance, engineered microorganisms in a

CBP system required longer fermentation periods than what is acceptable on an industrial scale. In addition, heterologous cellulolytic enzymes are secreted at low titres, as many enzymes are improperly folded or hyperglycosylated, rendering the enzymes non-functional. Furthermore, if high ethanol titres are obtained, the microorganism(s) exhibited low tolerance to the end-product and/or inhibitory components found in the pre-treated lignocellulosic hydrolysates used as feedstocks. Therefore, to address these challenges coupled to CBP implementation, a variety of improvements are required.

1.6. *Saccharomyces cerevisiae* as host for CBP strain construction

1.6.1. Characteristics of *Saccharomyces cerevisiae*

S. cerevisiae is widely used in various industries, such as brewing, baking, pharmaceuticals, and biofuels (Zhang *et al.,* 2022), as it enjoys generally regarded as safe (GRAS) status (Huang *et al.,* 2014b; Sheng *et al.,* 2017; Tippelt and Nett, 2021; Van Wyk *et al.,* 2018). Characteristics of *S. cerevisiae* of fundamental importance to industrial biofuel production includes fast growth in cheap media, high fermentation efficiency, ability to produce and tolerate high ethanol concentrations, high cell activity in acidic environments, osmo- and thermo-tolerance, and the ability to tolerate a wide variety of inhibitory conditions (Reis *et al.,* 2013; Xu *et al.,* 2014).

S. cerevisiae is used as chassis for industrial production of various recombinant proteins (Gast *et al.,* 2022). This is due to the yeasts' ability to perform post-translational modifications and its amenability to be genetically manipulated by various engineering tools, as it possesses efficient homologous recombination machinery that allows for stable integration of genetic elements (Gombert *et al.,* 2016; Gomes *et al.,* 2018; Van Wyk *et al.,* 2018). These characteristics and the wide array of information available regarding the yeasts' genome portrays it as a perfect candidate for use as a heterologous cell factory.

1.6.2. Expression of cellulase genes: strategies for expression

To date, several bacterial and fungal cellulase-encoding genes have been expressed in yeast species, as either (1) free enzymes, (2) cell-surface attached enzymes, or (3) through the construction of mini-cellulosomes (Figure 1.12) (Den Haan *et al.,* 2015; Lee *et al.,* 2017; Xu *et al.,* 2017). Each of these expression strategies have their own advantages and disadvantages. For instance, expression of cellulase-encoding genes for secretion as free enzymes has no limitation with regards to the physical surface area of the host cells, as individual cellulases are

released into the growth medium where it acts on the cellulosic substrates (Saini *et al.*, 2015). However, since most heterologous hosts tend to produce only low secretion yields, the hydrolysis process becomes hampered which results in low ethanol yields (Oh and Jin, 2020). In addition, diffusion of secreted enzymes away from the heterologous hosts makes recycling of enzymes for subsequent fermentations impossible (Den Haan *et al.*, 2015).

Cell-surface attached enzymes, on the other hand, are limited by the restricted physical surface area of their host and the potential display of inactive cellulases (Den Haan *et al.*, 2015). However, the proximity in which the cellulases are located to one another on the cell wall allows for a more efficient enzymatic hydrolysis, as detachment of enzymes from cellulosic substrates are alleviated, hence increasing the synergistic effect (Oh and Jin, 2020). Additionally, the potential for recycling of yeast cells for subsequent fermentations exists, without the need for further enzyme additions.

Similar to cell-surface attached cellulases, expression of cellulases in mini-cellulosomes is limited by the physical surface area of the host cell, and the post-translational modifications that the heterologous cellulases undergo may affect the correct display of cellulosomes on the microbial cell wall (Oh and Jin, 2020, Valenzuela-Ortega and French, 2019). However, the proximity in which cellulases are located to one another in cellulosomes contribute significantly to the efficiency of cellulose hydrolysis.

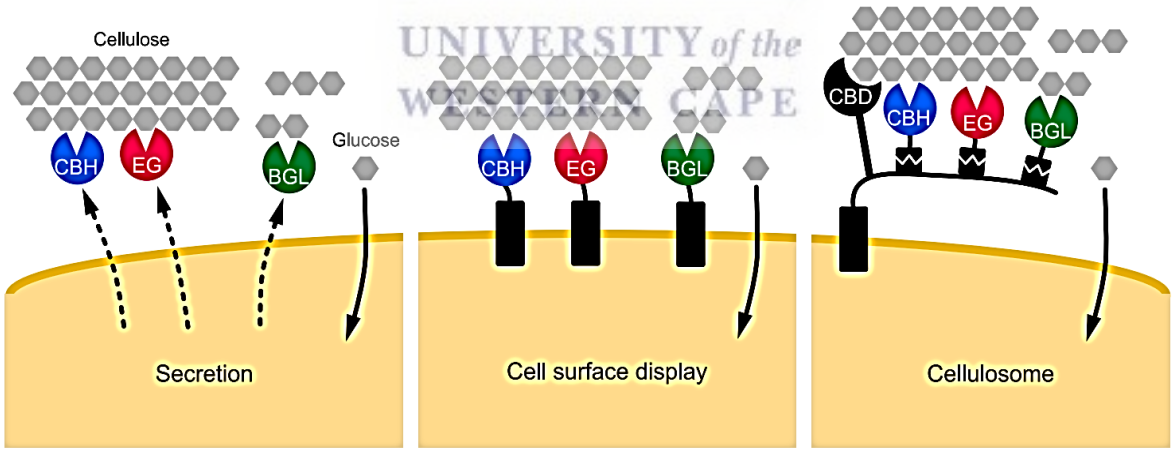

Figure 1.12: Strategies for cellulase gene expression in heterologous hosts. Heterologous cellulase production in recombinant hosts can be achieved via one of three strategies, namely, (1) secretion of cellulases as free enzymes to the extracellular environment where it then acts on the biomass substrate; (2) expressing cellulases as cell-surface attached enzymes to increase synergistic action, or (3) by expressing different cellulases as a complex in the form of a cellulosome. CBH, cellobiohydrolase; EG, endoglucanase; BGL, β-glucosidase (Oh and JIn, 2020).

Identifying a preferred strategy for heterologous cellulase expression remains problematic, due to insufficient data available (Den Haan *et al.,* 2015). Therefore, several research groups have explored each of the different strategies. Lee and co-workers (2017) accomplished secretion of four different cellulases, with the aid of an optimal translational fusion partner (TFP) that acted as both a fusion and secretion signal. Although variability was observed in the enzyme levels between recombinant yeasts, secretion levels of about 0.6-2.0 g/L were achieved. Furthermore, co-fermentation of the recombinant yeast strains on pre-treated rice straw produced ethanol yields up to 14 g/L from 35 g/L glucan contained in the biomass feedstock. These yields were 3-fold higher than that of the wild-type yeast strains, indicating a possible reduction in requirement for additional exogenous cellulase cocktails. In a similar study by Gong and co-workers (2014), endoglucanase and β-glucosidase from *T. viridae* were co-expressed for secretion from *S. cerevisiae*. Cultivation of recombinant *S. cerevisiae* on 20 g/L amorphous carboxymethyl cellulose (CMC), as sole carbon source, allowed for an ethanol yield of 4.63 g/L after 24 hours.

A study conducted by Hong and co-workers (2014) evaluated the ethanol-producing capabilities of *S. cerevisiae* strains particularly engineered for enhanced CBH activity. Using "δ" -sequence-mediated integration, recombinant strains were constructed by integrating cassettes containing constitutively expressed CBH-encoding genes (*CBHI* of *A. aculeatus*; *CBHI* and *CBHII* of *T. reesei*), *T. reesei EGII*, and *A. aculeatus BGLI*, to yield uni-, bi-, and trifunctional cellulase expression in resulting recombinant strains. Evaluating recombinant and control strains in acid and alkali pre-treated corncob containing media with a 5 filter paper unit (FPU) exogenous cellulase/g biomass loading, the highest ethanol titre (28.20(±0.84) g/L) was obtained for recombinant strain W3 after seven days. However, for the purpose of a CBP process, the addition of the commercial cellulase cocktail does not provide a clear indication as to the feasibility of using the W3 recombinant strain in a process without any exogenous enzyme additions.

A study conducted by Liu and co-workers (2016), successfully reported the display of four different cellulases on the cell surface of *S. cerevisiae* for direct ethanol production from cellulose. A previously engineered BGL-expressing *S. cerevisiae* strain was used as expression host for codon-optimised genes encoding *T. reesei* EGII, and two CBHs (I and II) from *Talaromyces emersonii* and *Chrysosporium lucknowense*, respectively. Constructing two recombinant *S. cerevisiae* strains containing BGL+EG+CBHI and BGL+EG+CBHII, respectively, allowed for ethanol titres of 2.3 g/L for both strain constructs when fermenting

amorphous phosphoric acid swollen cellulose (PASC). The combination of all four cellulases in a single *S. cerevisiae* construct however, significantly improved ethanol titres, reaching a maximum of 6.7 g/L when fermenting PASC. By combining individual cellulases required for efficient hydrolysis an increase in synergy between cellulases could be achieved, resulting in higher ethanol yields.

Chen and co-workers (2018) engineered a diploid *S. cerevisiae* strain to construct two types of cell-surface displayed strain variants for the heterologous expression of functional lignocellulolytic enzymes. Cellulase-encoding genes (*T. reesei eg* and *cbh*; and *A. aculeatus bgl*) or hemicellulase-encoding genes (*T. reesei zylA* and *A. oryzae xynII*) were expressed so that enzymes were displayed on *S. cerevisiae* Y5 with an α- agglutinin anchor, yielding strains Y5/EG-CBH-BGL and Y5/ZynII-ZylA. The feasibility of using these strains in a CBP consortium for cellulosic ethanol production, was tested with steam-exploded corn stover as feedstock. An ethanol titre of 1.61 g/L (64.7% of theoretical ethanol yield) was achieved after 144 hours of fermentation, without any addition of exogenous enzymes. Although this ethanol yield is relatively low, using a consortium in a CBP process does show some promise.

A direct comparison between cell-surface display and secretion as expression strategies for cellulases was conducted by Liu and co-workers (2015). Different recombinant *S. cerevisiae* strains were constructed containing the following combinations: (1) EG-D-CBHI-D and EG-S-CBHI-S (both enzymes were displayed on the surface, or both were secreted into the medium), and (2) EG-D-CBHI-S and EG-S-CBHI-D (EG displayed and CBHI secreted, or vice versa). The results obtained indicated that co-expressing synergistic cellulases on the cell surface of the heterologous host allowed for better cellulosic degradation when fermenting PASC, compared to when cellulases were secreted into the medium (ethanol titres of 2.9 and 2.6 g/L from 10 g/L PASC, respectively). However, employing a combined strategy, i.e., cell-surface display and secretion, yielded far less efficient ethanol production than employing secretion and/or cell-surface displaying, respectively. This may be due to blockage on the substrate surface caused by cell-surface attached cellulases, resulting in secreted cellulases not being able to access regions on the surface of the substrate. This comparison between cell-surface attachment and secretion of cellulases was in accordance with previous studies illustrating the effectiveness of cellulases in close proximity to one another to improve the efficiency of their synergy.

Since the re-construction of cellulosomes on the cell surface of *S. cerevisiae* is not as widely studied as the other two strategies, few comparable quantitative data sets are available (Den Haan *et al.*, 2013). However, Fan aand co-workers (2012) attempted re-construction of a mini-cellulosome containing the EG, CBH, and BGL, from *C. cellulolyticum* on the surface of *S. cerevisiae*. By growing the engineered strain on Avicel, the strain showed significant activity towards hydrolysing microcrystalline cellulose, yielding ethanol titres of up to 1.41 g/L. However, although the strain managed to convert cellulose to yield ethanol, most of the available cellulose was not degraded. This is likely due to low secretion titres and expression ratios of the enzymes; hence a low hydrolysis efficiency was achieved (Den Haan *et al.*, 2015).

As discussed, expression of cellulases with either of the three strategies has its own challenges and limitations. One consistent challenge is the generally low secretion phenotype observed in recombinant *S. cerevisiae* strains. To mitigate some of the challenges hampering the engineering of potential *S. cerevisiae* CBP strains, one could consider engineering strategies targeting protein secretory pathways in the yeasts, as well as the use of more naturally robust *S. cerevisiae* strains that may also have improved secretion phenotypes.

1.6.3. Protein secretory pathways of *S. cerevisiae*

The secretion of proteins is essential for normal function in all living organisms, whether single or multicellular (Delic *et al.*, 2013). In eukaryotes, proteins are synthesised in the cytosol, after which they are translocated across the endoplasmic reticulum (ER) membrane with the help of cytosolic chaperone proteins. The translocation process can occur either co-translationally (ribosome-coupled) or post-translationally (ribosome-uncoupled). Both translocation routes require a translocon pore, Sec61, in association with various channel partners, like the chaperone protein Kar2, for successful import of nascent proteins across the ER membrane. Once the translocated proteins enter the ER, the ER-Golgi pathway (Figure 1.13) is initiated, which is the default pathway for the secretion of proteins (Delic *et al.*, 2013; Xu *et al.*, 2014).

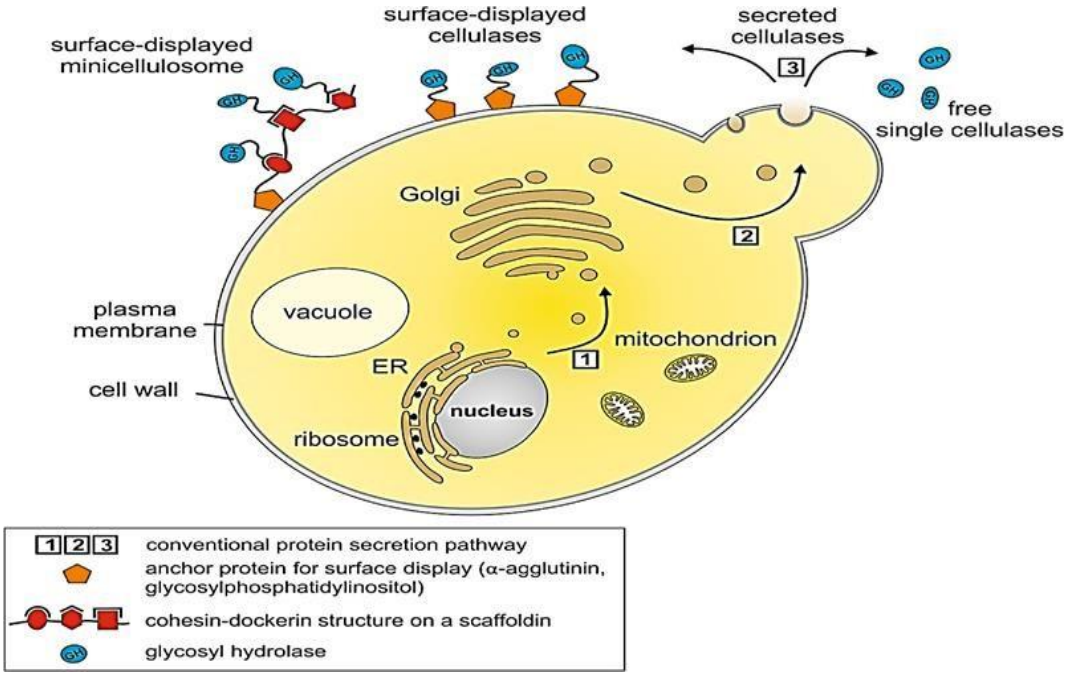

Figure 1.13: Schematic overview of the protein secretory pathway in yeasts. Protein synthesis in eukaryotic organisms is initiated in the cytosol, after which proteins are translocated to the endoplasmic reticulum (ER) where the ER-Golgi pathway is initiated. During this pathway, proteins undergo several covalent modifications (signal sequence processing, glycosylation, disulphide bond formation, sorting, etc), prior to exportation of properly folded proteins from the ER to the Golgi apparatus, where additional modifications occur before they are transported to other organelles or into the extracellular space (Lambertz *et al.,* 2014).

With the initiation of the ER-Golgi pathway, proteins undergo various covalent modifications, such as signal sequence processing, formation of disulphide bonds, N-terminus-glycosylation, and sorting (Delic *et al.,* 2013). Proteins entering the ER in their misfolded configuration are subjected to degradation and thus do not follow properly folded proteins through the entire ER-Golgi pathway to be secreted. The proteins that are properly folded and assembled are then exported from the ER to the Golgi apparatus where they undergo further modifications, before they are transported to their respective organelles or into the extracellular space (Delic *et al.,* 2013; Sheng *et al.,* 2017). In contrast, proteins in the ER lumen that are in their misfolded or unfolded configuration induce stress in the cells, which results in the activation of the Unfolded Protein Response (UPR) pathway. In this pathway, genes that are involved in proper folding and those involved in activation of the ER-associated degradation (ERAD) pathway are expressed. This results in misfolded proteins being diverted back to the cytosol where they are degraded by the ERAD pathway, or these proteins may be transported to vacuoles to be degraded by proteolytic enzymes (Delic *et al.,* 2013; Xu *et al.,* 2014). Even though such a sophisticated secretory pathway exists in *S. cerevisiae*, several bottlenecks halt its use as a cell factory for secreted heterologous proteins.

1.6.3.1. Bottlenecks in heterologous protein production in *S. cerevisiae*

With the overproduction of heterologous proteins, the energy demand for folding and glycosylation increases, which results in inducing cellular stress responses towards unfolded proteins (UPR) (Zahrl *et al.,* 2019). This response induces a significant burden on the metabolism of the yeast in terms of biomass yields and substrate consumption. Additionally, proteins that do manage to be post-translationally modified, often tend to be hyper-glycosylated and low secretion yields of properly folded proteins are obtained (Xu *et al.,* 2014). The low secretion yields may also be due to the absence of promoters with ideal transcriptional regulation and deregulation mechanisms, and the low gene copy numbers of the expressed heterologous genes (Zahrl *et al.,* 2019). Therefore, to overcome these challenges, several strategies can be employed to improve heterologous protein expression, both at the genetic and protein level.

1.6.3.2. Strategies for improvement of heterologous protein expression

Successful heterologous protein production is affected by several factors, including, selection of a specific host strain, properties of target proteins, expression vector systems, and cultivation conditions (Den Haan *et al.,* 2021). Furthermore, the steps that heterologous proteins undergo from expression of its genes to the secretion of functionally active proteins into the extracellular environment, play a pivotal role in the level of heterologously secreted proteins. In other words, challenges that exist during transcription, translation, or within post-translational modifications, are the determinantal factors for how much protein is secreted in a functionally active form. Although expression patterns of individual proteins are unique, several genetic and metabolic engineering approaches can be applied to overcome hurdles in each of the above-mentioned steps. For instance, low expression levels of heterologous protein-encoding genes can be mitigated by using expression plasmids with high copy numbers (Kroukamp *et al.,* 2018), or by integrating these genes into the genome of the recombinant hosts using CRISPR/Cas9 tools (Den Haan *et al.,* 2021). Additionally, choosing appropriate promoters and identifying codon usage preferences in hosts strains could also help ensure production of functional proteins (Ilmén *et al.,* 2011). However, since each heterologous protein has unique properties, identification of a general set of optimisation strategies is impossible.

1.7. Transformation approaches for gene integrations

Due to the constraints hampering the direct use of industrial and laboratory strains for CBP processes, several genetic engineering strategies have been employed to improve the efficiency

of recombinant yeasts (Dangi *et al.,* 2017). Conventional recombinant DNA methods have recently been boosted by advanced technologies such as systems biology, metabolomics, and gene editing using CRISPR/Cas9.

1.7.1. Traditional approaches

1.7.1.1. Plasmid vectors

The use of plasmid vectors for genetic engineering is an indispensable tool for the overexpression of heterologous genes in host cells, due to the ease with which the vectors can be manipulated and expression can be regulated (Li *et al.,* 2019; Da Silva and Srikrishnan, 2012). The number of plasmids available for use in *S. cerevisiae* is limited compared to those available for *Escherichia coli*, however, shuttle vectors that are autonomously replicating entities are commonly used. These include episomal plasmids (YEps), yeast centromeric plasmids (YCps) (Figure 1.14), and yeast integration plasmids (YIps), which can replicate in both *E. coli* and *S. cerevisiae* (Da Silva and Srikrishnan, 2012; Gnügge and Rudolf, 2017). For the stable maintenance of heterologous genes introduced via plasmids, auxotrophic markers are often used for increased selective pressure (Löbs *et al.,* 2017); however, other selection markers, such as auto-selection systems or dominant markers can also be utilised (Gnügge and Rudolf, 2017).

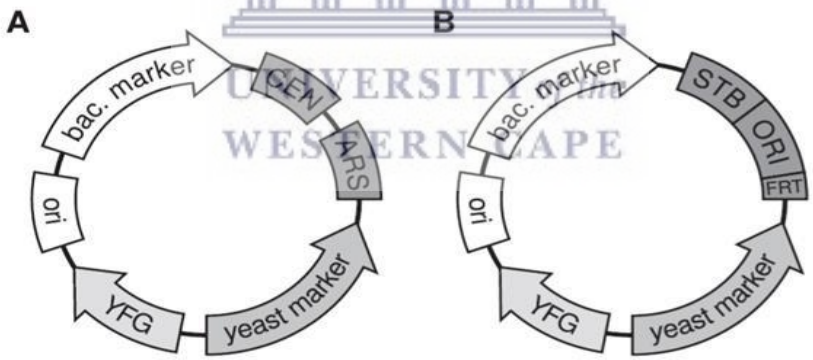

Figure 1.14: Traditional extrachromosomal yeast plasmids. (A) Yeast centromeric plasmid (YCp) containing a CEN and ARS sequences, and a selection marker. (B) Yeast episomal plasmid (YEp) containing STB and ORI sequences. YFG, your favorite gene; FRT, Flp1p recombinase site; bac. Marker, bacterial selection marker; ori, origin of replication; CEN, centromeric sequence; ARS, autonomously replicating sequence (Gnügge and Rudolf, 2017).

YCp vectors contain autonomous replication sequences (ARS) and centromeric (CEN) sequences from the *S. cerevisiae* genome to allow for their stable extrachromosomal maintenance in host cells (Zhang and An, 2010). These vectors have high transformation efficiency but are maintained at relatively low copy numbers of 1 to 10 copies per host cell

(Zhang and An, 2010; Zhang *et al.,* 1996). Therefore, for most applications requiring high expression levels, the more stable YEps are used. YEps are derived from the naturally occurring 2µ-plasmid found in *S. cerevisiae*, which allows higher stability with a copy number of up to 40 copies per cell (Zhang and An, 2010).

Although these plasmids are indispensable tools for genetic engineering, many disadvantages limit their use in large scale industrial production processes (Jensen *et al.,* 2014; Li *et al.,* 2019). The main problem associated with plasmids are their instability, in terms of structure and/or segregation, which can be caused by insertions/deletions or uneven partitioning to daughter cells in culture. Other disadvantages include the limitation in the available cloning sizes offered by plasmids, and the need to maintain selection pressure by culturing cells in defined media containing selection markers (Flagfeldt *et al.,* 2009; Jansen *et al.,* 2017; Li *et al.,*2019; Zhang *et al.,* 1996). To mitigate the problems associated with these extrachromosomal plasmids, a plasmid system was developed that allows for the integration of plasmids containing heterologous genes, into the genome of the host cells with a relatively high copy number (Sasaki *et al.,* 2019). Not only does this chromosomal integration approach allow for increased plasmid stability, but it also reduces the need for selection pressure, hence, reducing costs in industrial processes (Zhang *et al.,* 1996).

1.7.1.2. Chromosomal integration of genes

The integration of heterologous genes into host cell genomes is achieved with the use of YIps, at defined chromosomal loci (Löbs *et al.,* 2017). The two most widely used integrative vector systems used for multi-copy gene expression includes vectors aimed at integration at rDNA loci and into *δ* sequences (Semkiv *et al.,* 2016). The rDNA locus found in *S. cerevisiae* is about 1-2 mb in length, consisting of approximately 200 copies of 9.1 kb tandem repeat fragments (Semkiv *et al.,* 2016). Alternatively, δ-integration exploits the presence of long terminal repeats of *S. cerevisiae* retrotransposon (Ty1) elements dispersed throughout the yeast genome, by integrating target genes on multiple chromosomes, sometimes followed by crossing over between transformants of different mating types to allow for an increase in copy number of the integrated YIps (Gnügge and Rudolf, 2017). Despite the high number of available sites at which heterologous genes can be integrated using either rDNA or *δ*-sequences, these approaches are limited by genes only integrating at a few of these sites and the continuous need for selection pressure may still be required (Semkiv *et al.,* 2016). Although the

beforementioned approaches are still being employed, gene integration strategies have shifted to recombination events using DNA repair machinery.

Homologous recombination (HR) allows for the exchange of DNA between regions in the genome that contain identical sequences, so as to aid cellular DNA repair programs within cells (Li *et al.*, 2019). In genetic engineering, homologous recombination is therefore exploited to integrate heterologous genes of interest into the chromosomes of host cells for their expression. Homologous recombination can be achieved via single- or double crossover events (Figure 1.15), with the former being the most widely exploited, however it also depends on the number and the location at which target sequences will be integrated (Gnügge and Rudolf, 2017). With the single crossover recombination mechanism, single continuous target sequences are integrated at a specific locus in the host genome, allowing for the duplication of target sites in transformant cells. In contrast, the double crossover mechanism integrates multiple target sequences in a gene replacement manner, with a relatively high segregation stability (Gnügge and Rudolf, 2017). Using these integration approaches, selective pressure can be abandoned once the recombinant strain is constructed (Gomes *et al.*, 2018).

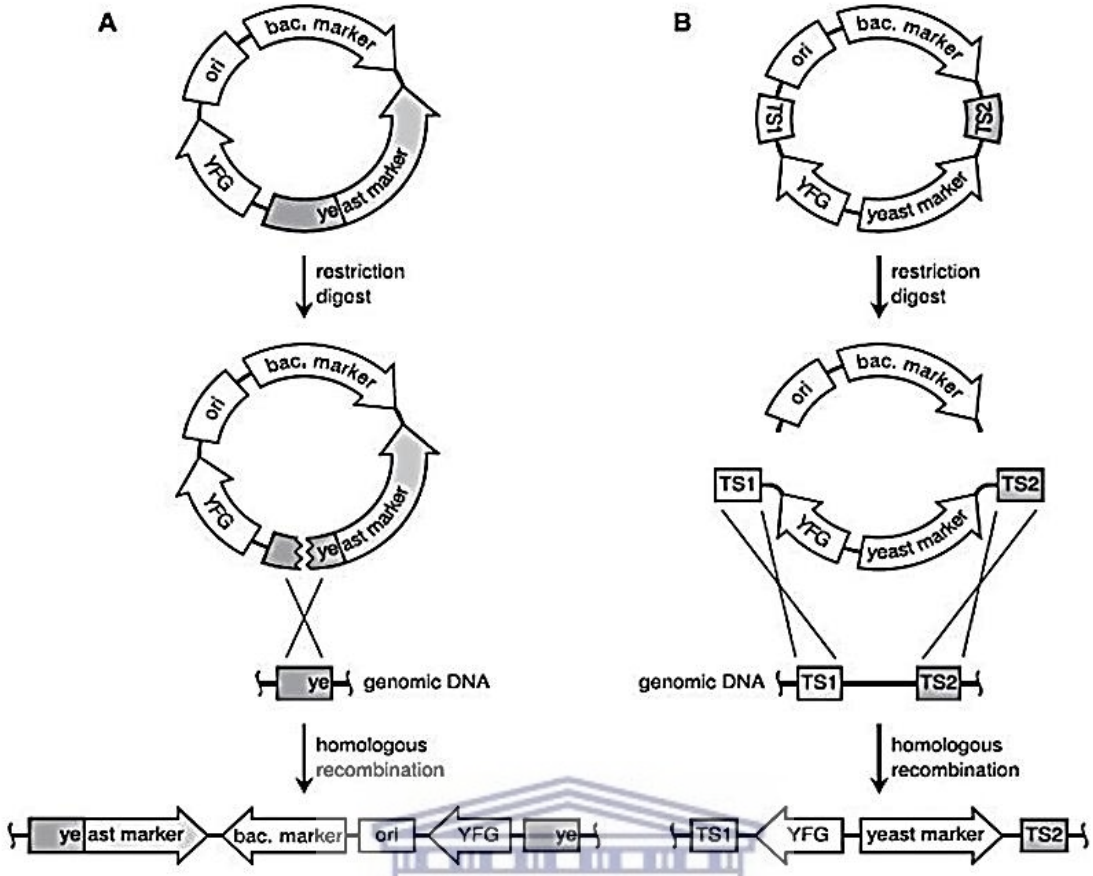

Figure 1.15: Yeast integrative plasmids used for homologous recombination, as either (A) single cross-over, or (B) double-crossover recombination mechanisms (Gnügge and Rudolf, 2017). YFG, your favorite gene; bac. marker, bacterial selection marker; ori, origin of replication; TS, targeting sequence.

Integrating heterologous genes into the genome of host cells through homologous recombination events, provides several advantages over the use of plasmid-based approaches (Jessop-Fabre *et al.,* 2016; Siddiqui *et al.,* 2014). For instance, integration of genes into the genome allows for improved strain stability and a lower degree of population heterogeneity. However, integration of selectable markers along with genes of interest is still inherent to the process, which leads to the problem of too few markers being available if multiple gene edits are required. To mitigate the problems associated with this limitation, marker recycling is required, which can be offered by the use of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology. This approach uses DNA endonucleases that introduce targeted double stranded breaks (DSBs) into the genomes of host cells.

1.7.2. CRISPR/Cas9 technology

The field of biology has been revolutionized in the last decade by the application of the prokaryotic adaptive defence system, referred to as CRISPR-associated endonuclease Cas9 (CRISPR/Cas9) (Figure 1.16) (Adiego-Pérez *et al.*, 2019; Javed *et al.*, 2019; Lino *et al.*, 2018). This system was described in bacterial and archael microorganisms as a defence mechanism against phage attack. The mechanism by which the attacks are countered is through the transcription of spacer sequences and palindromic repeats that yields a long RNA molecule. This molecule is then cleaved into small pieces (crRNA) with the help of trans-activating RNA (tracrRNA) and an endonuclease Cas9. The utilization of CRISPR/Cas9 as a gene editing tool has been made possible by the presence of sequences contained in crRNA that binds to tracrRNA to form a hairpin loop structure that can be cleaved by Cas9, as it is guided by the sequence contained in the crRNA.

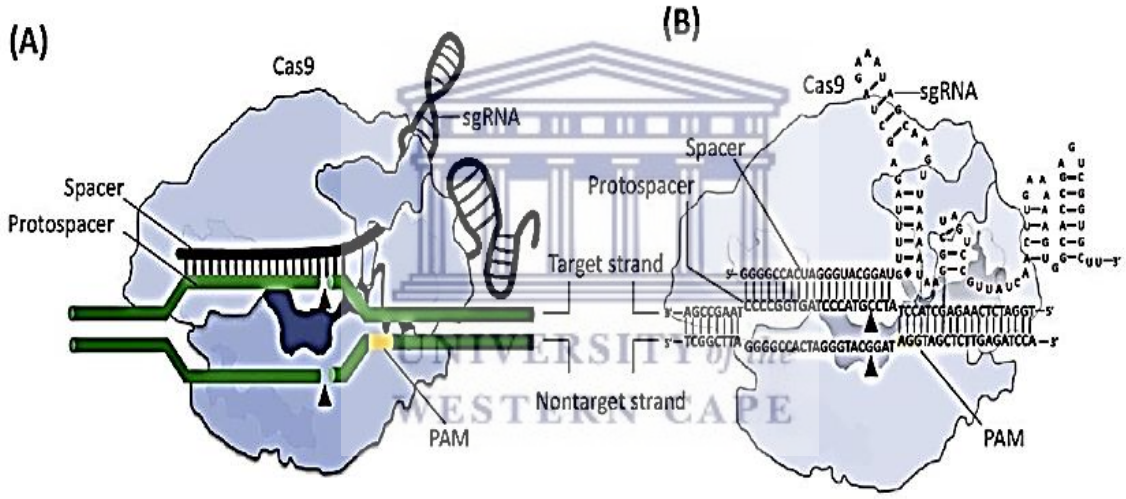

Figure 1.16: Cleavage of DNA using Cas9. (A) Graphic representation of Cas9 endonuclease in complex with single guide (sgRNA) cleaving a double stranded DNA molecule. (B) Inherent Cas9 endonuclease in complex with single guide (sgRNA) found as natural adaptive system in *Streptococcus pyogenes* (Donohoue *et al.*, 2018).

CRISPR/Cas9 as a gene editing tool is composed of two components: (1) an endonuclease Cas9 and (2) a small guide RNA (sgRNA) generated through the fusion of crRNA and tracrRNA from the endogenous bacteria from which it was isolated (Shanmugam *et al.*, 2020; Wolabu *et al.*, 2020). The Cas9 endonuclease generates double stranded breaks (DSBs) in the target DNA on the host genome, with the aid of the sgRNA that guides the Cas9 to the target sites. The sgRNA also allows for the hybridisation of RNA to DNA, as initiated between sgRNA and the

target DNA sequence. Following the target site at which hybridization occurs, a protospacer adjacent motif (PAM) sequence is located to ensure correct binding to the genomic DNA. Codon optimization and addition of a nuclear localization sequence has allowed the use of the *Streptococcus pyogenes* Cas9 in eukaryotes such as *S. cerevisiae.* In yeast, each of the CRISPR/Cas9 components are introduced into the recombinant host via plasmids. Once inside the host, sgRNA and the Cas9 protein forms a complex that allows for the localization of Cas9 to the target site where it generates a DSB in the host's genomic DNA. The presence of a DSB in the genome of an organism generally proves to be fatal to the survival of an organism, hence inherent DNA repair mechanisms are in place to repair such damage. In *S. cerevisiae*, DNA repair can be initiated using one of two mechanisms: (1) non-homologous end joining (NHEJ) or (2) homology-directed repair (HDR).

1.7.2.1. Non-homologous end-joining vs homology-directed repair

The preference for use of either NHEJ or HDR as DNA repair mechanism varies among organisms (EauClaire and Webb, 2019; Raschmanová *et al.,* 2018). With the introduction of a DSB as initiated by CRISPR-associated Cas9, the endogenous DNA repair machinery in microbial cells will be triggered. In the case where the HDR pathway is triggered, DNA from a sister chromatid is introduced at the site where the DSB occurred to direct repair. However, CRISPR/Cas9 as a gene editing tool can also allow for the supply of exogenous DNA to act as repair template. In doing so, new genes can be integrated into microbial genomes to confer new activities to the microorganism. In contrast, various microorganisms have an endogenous NHEJ pathway that is activated upon DNA damage. With this approach, break repairs occur independent of whether or not a repair template sequence was available. Thus, repair of DSBs often leads to the integration of insertions and/or deletions, referred to as random indels. The integration of random indels into genomic DNA may induce alterations to the functionality of the organism, which can ultimately threaten the survival of the microorganism. However, some organisms do have high infinity for the precise repair of DSB breaks with the NHEJ pathway, such as *Kluyveromyces marxianus* (Hoshida *et al.,* 2013). Understanding which pathway an organism has high affinity for generally makes genetic engineering using CRISPR/Cas9 technology more feasible.

1.7.2.2. Use of CRISPR/Cas9 technology in *S. cerevisiae* and fungal microorganisms

Fungi have huge biotechnological value as they produce large quantities of valuable compounds that are useful in industrial processes (Schuster and Kahmann, 2019). To meet the demand for these valuable compounds, CRISPR/Cas9 gene editing can be used to exploit the production pathways within these organisms. As shown by Liu and co-workers (2017), thermophilic strains of *Myceliophthora* sp. could be engineered using CRISPR/Cas9 technology for hyper-cellulase secretion by integrating mutations in the *amdS* gene at different loci via NHEJ. This genetic engineering allowed for a 5-fold increase in the level of extracellular secreted proteins. In addition, a 13-fold increase in the lignocellulolytic activity of these fungal strains could also be achieved. Similarly, Matsu-ura and co-workers (2015) have illustrated the use of CRISPR/Cas9 technology in *Neurospora crassa* species. The study evaluated the utilisation of CRISPR/Cas9 to replace the endogenous *clr-2* promoter with the beta-tubulin promoter for increased expression of cellulases by the fungal species. The study found an approximate 200-fold increase in *clr-2* mRNA expression when under transcriptional control of the beta-tubulin promoter. In addition, CRISPR/Cas9 could also be used to steer homology-directed repair in organisms lacking NHEJ pathways. Nødvig and co-workers (2018) showed that a single stranded oligonucleotide sequence could be used as repair template to repair the DSB generated by the Cas9/sgRNA in a NHEJ-deficient *Aspergillus* sp. with an efficiency of ~100%. These examples show that, fungal organisms could be optimised for improved production of valuable compounds necessary for industrial applications, with CRISPR/Cas9 as gene editing tool.

The highly efficient homologous recombination pathway in *S. cerevisiae* allows for the introduction of multiple changes into its genome using CRISPR/Cas9 (Stovicek *et al.*, 2017). A study conducted by Xu and co-workers (2018) illustrated the use of CRISPR/Cas9 to mediate a gene knock-out within *S. cerevisiae*. The introduction of a frameshift mutation at the *ADH2* locus allowed for an accurate gene editing efficiency of 91.4%. Subjecting the *ADH2*-deficient *S. cerevisiae* strain to bioethanol production allowed for an optimisation in the ethanol yield of up to 74.7% in comparison with the parental strain. Similarly, Liu and co-workers (2019) successfully introduced three gene disruptions in a single step with high efficiency using CRISPR/Cas9 technology. The three genes (*ADH2*, *GPD1*, and *ALD4*) involved in the ethanol production pathway of *S. cerevisiae* were disrupted individually and in combination to identify isolates with higher ethanol production efficiencies. Disruption efficiencies ranging from 80 to

100% for single and combinatory disruptions were reported. Isolates were also identified with an approximate 1.41-fold higher ethanol production efficiency compared to the wild-type.

1.7.2.3. Advantages and disadvantages of CRISPR/Cas9 technology compared to traditional engineering approaches

The introduction of CRISPR/Cas9 technology has revolutionised the genome editing platform with several benefits lacked by conventional genome editing tools (Raschmanová *et al.*, 2018). The introduction of sequences into genomes in a marker-less manner has greatly benefitted research by reducing the time needed for counter-selection and marker recycling. In addition, using this sophisticated engineering tool allowed for the introduction of single point mutations, deleting whole genes, and integrating entire new gene sequences at defined loci within genomes. Further optimisation of this editing tool over the past decade has also allowed for introducing multiple disruptions to genes in a single step. Compared to the traditional engineering tools available for genetic engineering in organisms, CRISPR/Cas9 has greater target specificity, is versatile and precise, and is easy to use at relatively low expense (Wolabu *et al.*, 2020). However, despite its merits, CRISPR/Cas9 also holds many limitations as a genetic editing tool. As reviewed by Peng and co-workers (2016), the activity of Cas9 and the experimental design of sgRNA for the selection of target genomic sites greatly influences the specificity and efficiency at which CRISPR/Cas9 systems work in hosts. Other limitations include off-target effects, the incidence at which homologous recombination occur, and the manner in which CRISPR plasmids are delivered to the host cells. Influencing the efficiency and specificity of Cas9 and sgRNA greatly affects the recognition of target sites at which DSBs will be introduced. The presence of mutations at or near the PAM sequences recognised by sgRNA precludes binding to target sequences resulting in no cleavage by the Cas9 endonuclease. This occurrence will lead to no genome editing taking place. Although CRISPR/Cas9 technology holds many benefits over conventional plasmid-based approaches in creating process ready host strains, these concerns must be considered.

1.8. Industrial and/or natural isolates for use in CBP

The genetic and phenotypic diversity of strains is greatly dependent on the environmental conditions from which strains are isolated (Steensels *et al.*, 2014). In the case of industrial yeast strains, adaptations to their environments confers specific genetic signatures to strain variants, that are characteristic of a particular industrial application. Although these adaptation events have allowed strains to evolve into more robust hosts for their application, the genomic

rearrangements that have brought about these changes yield strain variants with less phenotypic and genetic variation. This results in strains with (1) less heterozygosity compared to natural strain variants, (2) less sequence diversity, (3) high variation in ploidy (often aneuploid and/or polyploid), and (4) high genome structural variation (Davison *et al.*, 2020; Hose *et al.*, 2020; Molinet and Cubillos, 2020; Steensels *et al.*, 2014; Steensels and Verstrepen, 2014). As a result, utilisation of domesticated industrial strains tends to exhibit reduced robustness and cellular fitness after several seasons of bioethanol production, due to strains struggling to adapt to continuous changes in the fermentation processes. This phenomenon is emphasised in Brazilian 1G bioethanol production, using sugarcane bagasse (reviewed by Della-Bianca *et al.*, 2013). Therefore, improving existing industrial strains or identifying novel natural strains are required to ensure a sustainable future for bioethanol production.

Compared to 1G bioethanol production from corn and sugarcane, 2G bioethanol production in a CBP setting require strains capable of thriving in fluctuating conditions of a harsh nature (Fernandes *et al.*, 2022; Steensels *et al.*, 2014; Steensels and Verstrepen, 2014). While strains should have the ability to produce the cellulolytic enzymes necessary for cellulosic hydrolysis, pathways for both pentose and hexose sugars should be available in these hosts to ensure maximum assimilation of fermentable sugars to obtain high ethanol yields. However, selection of appropriate hosts for the bioethanol production process is greatly dependent on the tolerance of hosts towards stresses found in the fermentation process. Therefore, several studies have evaluated quantitative traits among natural and domesticated industrial strains with the aim to identify organisms for use in CBP bioethanol processes (Davison *et al.*, 2020).

A study undertaken by Gronchi and co-workers (2022) demonstrated the potential use of the natural vineyard isolate, *S. cerevisiae* L20, in bioethanol production from starch. Engineering the natural isolate for expression of *α*-amylase *amyA* and glucoamylase *glaA* genes from *A. tubingensis* allowed for an ethanol yield of 4 g/L from 2% starch, without the addition of any exogenous amylolytic enzymes. Although equal copies of the amylase-encoding genes were present in this isolate compared with the reference Ethanol Red strain, the recombinant natural isolate yielded significantly higher amylolytic activity. In a similar study, Favaro and co-workers (2013) explored the diversity of natural strains indigenous to grape marc for their potential to exhibit increased tolerance to high ethanol and inhibitory conditions. Testing 40 newly identified natural isolates showed their potential to produce high ethanol yields when cultivated at 40°C in minimal media supplemented with high glucose concentrations of up to 200 g/L, as well as exhibiting increased tolerance profiles against varying inhibitor

concentrations. Evaluation of these newly identified natural isolates, in comparison with industrial and commercial bioethanol strains (MH1000, 27P, EC1118, and DSM70449), demonstrated superior fermentation profiles and increased tolerance by the natural isolates when subjected to fermentation of liquor from steam pre-treated sugarcane bagasse. Superior ethanol concentrations of up to 43.4 g/L, corresponding to 89% of the theoretical maximum yield was obtained by *S. cerevisiae* Fm17, illustrating the potential of this natural isolate as a chassis organism in CBP processes for bioethanol production.

As illustrated in the abovementioned examples, natural isolates of *S. cerevisiae* offer many advantageous properties over that of commercial and industrial strains for use as potential CBP hosts for 1G bioethanol production. However, due to the complexity of inhibitory compounds and conditions present in lignocellulosic bioethanol production, yeast strains with more robust backgrounds are required for development of 2G industrial strain platforms (Jansen *et al.*, 2017). For this reason, Davison and co-workers (2016) focussed on screening/identifying natural robust *S. cerevisiae* strains from various vineyards along the coastal regions of the Western Cape, South Africa, for improved heterologous cellulase secretion and/or ethanol yields and titres, whilst performing in an inhibitory environment.

Using episomal plasmids, three cellulase encoding genes (*Saccharomycopsis fibuligera S.f.cel3A* – BGL; *T. reesei T.r.cel5A* -EG; and *T. emersonii T.e.cel7A* – CBH) were individually transformed into natural *S. cerevisiae* isolates (Davison *et al.*, 2016). Evaluating β-glucosidase (BGL) activity in the transformants containing the *S.f.cel3A* expressed gene, *S. cerevisiae* FIN1 yielded the highest extracellular activity (2.54 U/mg dry cell weight (DWC)) which corresponded to a 21-fold higher enzyme activity compared to the reference strain *S. cerevisiae* S288c. Strains MF15, YI19 and YI59 also presented high extracellular activity, however at a lower rate to that of FIN1. Transformants with moderate extracellular activity included YI13, YI1, and V3. Furthermore, transformants expressing the endoglucanase (*T.r.cel5A*) and cellobiohydrolase (*T.e.cel7A*) genes were also screened for superior secretors. Although FIN1 showed relatively high enzyme activity for the BGL, relatively low to moderate enzyme activities were obtained for EG and CBH, respectively. However, the moderate BGL secretor, YI13, demonstrated the highest extracellular enzyme activity for both EG and CBH. Extracellular enzyme activities of 6.50 U/mg DCW and 9.99 U/mg DCW corresponding to a 3.7 and 3.5-fold higher activity, respectively, compared to that of reference S288c transformed strain.

To evaluate the potential of transformants to secrete these cellulase enzymes under oxygen limiting conditions whilst still producing high ethanol titres, transformants were subjected to simulated fermentation conditions (Davison *et al.*, 2016). The findings demonstrated that YI13 yielded high ethanol production titres (9.02 g/L and 88% theoretical yield) compared to the highest ethanol producer, the industrial strain MH1000 (9.09 g/L and 88% theoretical yield). In addition, YI13 also demonstrated the fastest glucose consumption rate with residual glucose levels of ~0.3 g/L compared to the other transformants. YI13 was thus superior in its ability to secrete enzymes at relatively high levels compared to the reference S288c. In further evaluation based on transformant strain inhibitor tolerance, YI13 and MH1000 showed the highest tolerance to NaCl, heat, ethanol, and other inhibitor-stress. In addition, YI13 also showed relatively high tolerance to Tunicamycin at concentrations of 0.8-1.0 µg/ml. Based on the findings obtained in this study, it was clearly shown that YI13 was a superior natural isolate. With its increased robustness and secretion abilities, this isolate has great potential for use in construction of a model CBP organism for use in bioethanol production processes operated under harsh conditions.

1.9. Aim and objectives of this study

Based on the literature reviewed, significant progress has been made in developing more sustainable processes to produce renewable energy sources, such as bioethanol. However, several challenges still hamper the implementation of production processes that are sustainable but also cost-efficient. *S. cerevisiae* is known for its ability to yield high ethanol titres, whilst performing in harsh fermentation environments and has received great attention across several platforms due to the ease with which it can be manipulated. While traditional genetic engineering approaches using plasmid-vectors are still the norm amongst researchers, the implementation of gene editing tools like CRISPR/Cas9 allows a more efficient and marker-less means to manipulate *S. cerevisiae* for bioethanol production.

Therefore, in this study we aimed to apply CRISPR/Cas9 to introduce genetic modifications into natural *S. cerevisiae* strains isolates previously identified. By endowing these strains with a basic cellulolytic system and optimising strain performance, it was hoped that improved cellulose CBP strains could be obtained for application in 2G bioethanol production. To attain this aim we pursued the following objectives:

- Transformation of natural *S. cerevisiae* isolates (YI13, FIN1, and YI59) and an industrial reference strain (MH1000) with a full set of cellulase complex genes (*EGII, CBHI, CBHII,* and *BGLI*) using CRISPR/Cas9 technology
- Evaluate enzyme activity levels of secreted cellulases in various fermentation conditions, namely, elevated temperatures or in the presence of acetic acid
- Analyse metabolic burden exerted on yeast cells due to expression and secretion of the heterologous cellulolytic enzymes, under the same conditions
- Screen transformed strain isolates for robustness against inhibitory conditions commonly associated with general bioethanol fermentation processes, and secretion and/or cell wall stress
- Evaluate the strains' ability to convert microcrystalline cellulose (Avicel) to ethanol in a CBP configuration without the addition of exogenous enzymes.

CHAPTER 2: MATERIALS AND METHODS

2.1. Microbial strains, plasmids, and PCR sequences

All microbial strains and plasmids utilised in this study are listed in Table 2.1 and Table 2.2. One reference strain, namely the previously described industrial strain *S. cerevisiae* MH1000 was included for comparison of strain background diversity. For the evaluation of enzyme activity levels, three variants of *S. cerevisiae* Y294 were also included, where each variant expressed one of the cellulase complex genes.

Table 2.1: Microbial strains used in this study

Microbial strain	Abbreviation	Description	Reference
Parental strains			
Saccharomyces cerevisiae YI13	YI13_WT	Natural isolate	Davison *et al.*, (2016)
S. cerevisiae FIN1	FIN1_WT	Natural isolate	Davison *et al.*, (2016)
S. cerevisiae YI59	YI59_WT	Natural isolate	Davison *et al.*, (2016)
S. cerevisiae MH1000	MH1000_WT	Diploid industrial strain with no auxotrophy	Davison *et al.*, (2016)
Constructed strains			
S. cerevisiae YI13 + pCas9 + (pRS42-G-ChX + *ENO1*$_P$-*T.r.EGII-ENO1*$_T$) + (pRS42-G-Δ + *ENO1*$_P$-*T.e.CBHI-ENO1*$_T$) + (pRS42-G-ChXI + *ENO1*$_P$-*S.f.BGLI-ENO1*$_T$)	YI13_ECBE	YI13_WT transformed with CRISPR plasmids, pCas9-Nat and gRNA plasmids (pRS42-G-ChX, pRS42-G-Δ, and pRS42-G-ChXI, respectively), and homology repair templates *T.r.EGII*, *T.e.CBHI*, and *S.f.BGLI*, respectively, in successive rounds of transformation.	This study
S. cerevisiae FIN1 + pCas9 + (pRS42-G-ChX + *ENO1*$_P$-*T.r.EGII-ENO1*$_T$) + (pRS42-G-Δ + *ENO1*$_P$-*T.e.CBHI-ENO1*$_T$) + (pRS42-G-ChXI + *ENO1*$_P$-*S.f.BGLI-ENO1*$_T$)	FIN1_ECBE	FIN1_WT transformed with CRISPR plasmids, pCas9-Nat and gRNA plasmids (pRS42-G-ChX, pRS42-G-Δ, and pRS42-G-ChXI, respectively), and homology repair templates *T.r.EGII*, *T.e.CBHI*, and *S.f.BGLI*, respectively, in successive rounds of transformation.	This study
S. cerevisiae YI59 + pCas9 + (pRS42-G-ChX + *ENO1*$_P$-*T.r.EGII-ENO1*$_T$) + (pRS42-G-Δ + *ENO1*$_P$-*T.e.CBHI-ENO1*$_T$) + (pRS42-G-ChXI + *ENO1*$_P$-*S.f.BGLI-ENO1*$_T$)	YI59_ECBE	YI59_WT transformed with CRISPR plasmids, pCas9-Nat and gRNA plasmids (pRS42-G-ChX, pRS42-G-Δ, and pRS42-G-ChXI, respectively), and homology repair templates *T.r.EGII*, *T.e.CBHI*, and *S.f.BGLI*, respectively, in successive rounds of transformation.	This study

S. cerevisiae MH1000 + pCas9 + (pRS42-G-ChX + *ENO1*$_P$-*T.r.EGII-ENO1*$_T$) + (pRS42-G-Δ + *ENO1*$_P$-*T.e.CBHI-ENO1*$_T$) + (pRS42-G-ChXI + *ENO1*$_P$-*S.f.BGLI-ENO1*$_T$)	MH1000_ECBE	MH1000_WT transformed with CRISPR plasmids, pCas9-Nat and gRNA plasmids (pRS42-G-ChX, pRS42-G-Δ, and pRS42-G-ChXI, respectively), and homology repair templates *T.r.EGII*, *T.e.CBHI*, and *S.f.BGLI*, respectively, in successive rounds of transformation.	This study
S. cerevisiae YI13 + pCas9 + (pRS42-G-ChX + *ENO1*$_P$-*T.r.EGII-ENO1*$_T$) + (pRS42-G-Δ + *ENO1*$_P$-*T.e.CBHI-ENO1*$_T$) + (pRS42-G-ChXI + *PGK1*$_P$-*S.f.BGLI-PGK1*$_T$)	YI13_ECBP	YI13_WT transformed with CRISPR plasmids, pCas9-Nat and gRNA plasmids (pRS42-G-ChX, pRS42-G-Δ, and pRS42-G-ChXI, respectively), and homology repair templates *T.r.EGII*, *T.e.CBHI*, and *S.f.BGLI*, respectively, in successive rounds of transformation.	This study
S. cerevisiae FIN1 + pCas9 + (pRS42-G-ChX + *ENO1*$_P$-*T.r.EGII-ENO1*$_T$) + (pRS42-G-Δ + *ENO1*$_P$-*T.e.CBHI-ENO1*$_T$) + (pRS42-G-ChXI + *PGK1*$_P$-*S.f.BGLI-PGK1*$_T$)	FIN1_ECBP	FIN1_WT transformed with CRISPR plasmids, pCas9-Nat and gRNA plasmids (pRS42-G-ChX, pRS42-G-Δ, and pRS42-G-ChXI, respectively), and homology repair templates *T.r.EGII*, *T.e.CBHI*, and *S.f.BGLI*, respectively, in successive rounds of transformation.	This study
S. cerevisiae YI59 + pCas9 + (pRS42-G-ChX + *ENO1*$_P$-*T.r.EGII-ENO1*$_T$) + (pRS42-G-Δ + *ENO1*$_P$-*T.e.CBHI-ENO1*$_T$) + (pRS42-G-ChXI + *PGK1*$_P$-*S.f.BGLI-PGK1*$_T$)	YI59_ECBP	YI59_WT transformed with CRISPR plasmids, pCas9-Nat and gRNA plasmids (pRS42-G-ChX, pRS42-G-Δ, and pRS42-G-ChXI, respectively), and homology repair templates *T.r.EGII*, *T.e.CBHI*, and *S.f.BGLI*, respectively, in successive rounds of transformation.	This study
S. cerevisiae MH1000 + pCas9 + (pRS42-G-ChX + *ENO1*$_P$-*T.r.EGII-ENO1*$_T$) + (pRS42-G-Δ + *ENO1*$_P$-*T.e.CBHI-ENO1*$_T$) + (pRS42-G-ChXI + *PGK1*$_P$-*S.f.BGLI-PGK1*$_T$)	MH1000_ECBP	MH1000_WT transformed with CRISPR plasmids, pCas9-Nat and gRNA plasmids (pRS42-G-ChX, pRS42-G-Δ, and pRS42-G-ChXI, respectively), and homology repair templates *T.r.EGII*, *T.e.CBHI*, and *S.f.BGLI*, respectively, in successive rounds of transformation.	This study

S. cerevisiae YI13 + pCas9 + (pRS42-G-ChX + *ENO1*$_P$-*T.r.EGII-ENO1*$_T$) + (pRS42-G-Δ + *ENO1*$_P$-*T.e.CBHI-ENO1*$_T$) + (pRS42-G-ChXI + SED1$_P$-*A.a.BGLI*-DIT1$_T$) + (pRS42-G-Δ + *ENO1*$_P$-*C.l.CBHII-ENO1*$_T$)	YI13_BECC	YI13_WT transformed with CRISPR plasmids, pCas9-Nat and gRNA plasmids (pRS42-G-ChX, pRS42-G-Δ, and pRS42-G-ChXI, respectively) and homology repair templates *T.r.EGII*, *T.e.CBHI*, *C.l.CBHII*, and *A.a.BGLI*, respectively, in successive rounds of transformation.	This study
S. cerevisiae FIN1 + pCas9 + (pRS42-G-ChX + *ENO1*$_P$-*T.r.EGII-ENO1*$_T$) + (pRS42-G-Δ + *ENO1*$_P$-*T.e.CBHI-ENO1*$_T$) + (pRS42-G-ChXI + SED1$_P$-*A.a.BGLI*-DIT1$_T$) + (pRS42-G-Δ + *ENO1*$_P$-*C.l.CBHII-ENO1*$_T$)	FIN1_BECC	FIN1_WT transformed with CRISPR plasmids, pCas9-Nat and gRNA plasmids (pRS42-G-ChX, pRS42-G-Δ, and pRS42-G-ChXI, respectively) and homology repair templates *T.r.EGII*, *T.e.CBHI*, *C.l.CBHII*, and *A.a.BGLI*, respectively, in successive rounds of transformation.	This study
S. cerevisiae YI59 + pCas9 + (pRS42-G-ChX + *ENO1*$_P$-*T.r.EGII-ENO1*$_T$) + (pRS42-G-Δ + *ENO1*$_P$-*T.e.CBHI-ENO1*$_T$) + (pRS42-G-ChXI + *SED1*$_P$-*A.a.BGLI-DIT1*$_T$) + (pRS42-G-Δ + *ENO1*$_P$-*C.l.CBHII-ENO1*$_T$)	YI59_BECC	YI59_WT transformed with CRISPR plasmids, pCas9-Nat and gRNA plasmids (pRS42-G-ChX, pRS42-G-Δ, and pRS42-G-ChXI, respectively) and homology repair templates *T.r.EGII*, *T.e.CBHI*, *C.l.CBHII*, and *A.a.BGLI*, respectively, in successive rounds of transformation.	This study
S. cerevisiae MH1000 + pCas9 + (pRS42-G-ChX + *ENO1*$_P$-*T.r.EGII-ENO1*$_T$) + (pRS42-G-Δ + *ENO1*$_P$-*T.e.CBHI-ENO1*$_T$) + (pRS42-G-ChXI + SED1$_P$-*A.a.BGLI*-DIT1$_T$) + (pRS42-G-Δ + *ENO1*$_P$-*C.l.CBHII-ENO1*$_T$)	MH1000_BECC	MH1000_WT transformed with CRISPR plasmids, pCas9-Nat and gRNA plasmids (pRS42-G-ChX, pRS42-G-Δ, and pRS42-G-ChXI, respectively) and homology repair templates *T.r.EGII*, *T.e.CBHI*, *C.l.CBHII*, and *A.a.BGLI*, respectively, in successive rounds of transformation.	This study
Reference strains (Enzyme activity)			
S. cerevisiae Y294 + pRDH147::*fur1*	Y294_EGII	*S. cerevisiae* Y294 containing pRDH147 (*T.r.EGII* gene under control of *ENO1*$_P$ and *ENO1*$_T$), and a disrupted *fur1* (*fur1::LEU2*)	Brevnova *et al.*, (2011)

S. cerevisiae Y294 + pMI529::*fur1*	Y294_CBHI	*S. cerevisiae* Y294 containing pMI529 (*T.e.CBHI* gene under control of $ENO1_P$ and $ENO1_T$), and a disrupted *fur1* (*fur1::LEU2*)	Davison *et al.*, (2016)
S. cerevisiae Y294 + ySFI::*fur1*	Y294_BGLI	*S. cerevisiae* Y294 containing ySFI (*S.f.BGLI* under control of $PGK1_P$ and $PGK1_T$), and a disrupted *fur1* (*fur1::LEU2*)	Davison *et al.*, (2016)

The plasmids containing the homology repair gene cassettes, the *cas9* expression cassette, and gRNA target regions, respectively, are listed in Table 2.2.

Table 2.2: Plasmids used in this study

Plasmid	Description	Reference
pRDH180	Plasmid containing $ENO1_P$ and $ENO1_T$, and *T.r.EGII* gene cassette (used to produce homology repair template DNA for *T.r.EGII* integration)	Brevnova *et al.*, (2011)
pMI529	Plasmid containing $ENO1_P$ and $ENO1_T$, and *T.e.CBHI* gene cassette (used to produce homology repair template DNA for *T.e.CBHI* integration)	Ilmén *et al.*, (2011)
pMU-*BGLI*	Plasmid containing $ENO1_P$ and $ENO1_T$, and *S.f.BGLI* gene cassette (used to produce homology repair template DNA for $ENO1_P$-*S.f.BGLI*-$ENO1_T$ integration)	Davison *et al.*, (2019)
ySFI	Plasmid containing $PGK1_P$ and $PGK1_T$, and *S.f.BGLI* gene cassette (used to produce homology repair template DNA for $PGK1_P$-*S.f.BGLI*-$PGK1_T$ integration)	Van Rooyen *et al.*, (2005)
pMU784	Plasmid containing $PGK1_p$ and $PGK1_T$, and *C.l.CBHII* gene cassette (used to produce homology template DNA for *C.l.CBHII* integration)	Ilmén *et al.*, (2011)
pIBG-SSAD	Plasmid containing $SED1_P$ and $DIT1_T$, and *A.a.BGLI* gene cassette (used to produce homology template DNA for $SED1_P$-*A.a.BGLI*-$DIT1_T$ integration)	Inokuma *et al.*, (2021)
pCas9-Nat	Plasmid with *cas9* expression cassette	ADDGENE
pRS42-G-ChX	gRNA scaffold plasmid that targets Chromosome 10 intergenic region	Jacob (2022)
pRS42-G-ChXI	gRNA scaffold plasmid that targets Chromosome 11 intergenic region	Kruger and Den Haan (2022)
pRS42-G-Δ	gRNA scaffold plasmid that targets delta sequences within the yeast chromosome	Jacob (2022)

The primers used for the amplification of the gene cassettes and confirmation of integration are detailed in Table 2.3. For all PCR amplifications performed, RedTaq™ DNA polymerase (Ampliqon, Odense, Denmark) or OneTaq™ DNA polymerase (New England Biolabs,

Ipswich, MA, USA) was used according to the manufacturer's instructions, in an Applied Biosystems Thermocycler.

Table 2.3: Primers used for the amplification and confirmation of gene cassettes and sgRNA plasmids

Primer name	Sequence (5'- 3')	Application
Ch10.ENO1$_P$-L	GCAGTTATCTCTGTGTCCAGATCCCTT	Amplify homology repair template DNA (*ENO1$_P$-T.r.EGII-ENO1$_T$*) with Chromosome 10 target homology
Ch10.ENO1$_T$-R	CTACAGTAATTGTGCGGTGCAGGGAGG	
DELTA-ENO1-L	CTTAAGATGCTCTTCTTATTCTATTAAAAA TAGAAAATGACTTCTAGGCGGGTTATCTA CTG	Amplify homology repair template DNA (*ENO1$_P$-T.e.CBHI-ENO1$_T$*) with Delta (Δ) target homology
DELTA-ENO1-R	GTTTGTTTGCGAAACCCTATGCTCTGTTGT TCGGATTTGACGTCGAACAACGTTCTATT AGG	
DELTA_PGK1p-L	CTTAAGATGCTCTTCTTATTCTATTAAAAA TAGAAAATGATCCCTCCTTCTTGAATTG	Amplify homology repair template DNA (*PGK1$_P$-C.l.CBHII-PGK1$_T$*) with Delta (Δ) target homology
DELTA_PGK1p-R	GTTTGTTTGCGAAACCCTATGCTCTGTTGT TCGGATTTGAAACGCAGAATTTTCGAG	
Chr11 int-ENO-L	TGTAAAACAGGTATTGGCTGCTTCATAGT ACACCCAATTGCTTCTAGGCGGGTTATCT ACTG	Amplify homology repair template DNA (*ENO1$_P$-S.f.BGLI-ENO1$_T$*) with Chromosome 11 target homology
Chr11 int-ENO-R	GCAACTCTGAAATGTCAAAACGGTCGTGT ATAAATAAATGCCGTCGAACAACGTTCTA TTAGG	
Chr11 int-PGK-L	TGTAAAACAGGTATTGGCTGCTTCATAGT ACACCCAATTGTCCCTCCTTCTTGAATTG	Amplify homology repair template DNA (*PGK1$_P$-S.f.BGLI-PGK1$_T$*) with Chromosome 11 target homology
Chr11 int-PGK-R	GCAACTCTGAAATGTCAAAACGGTCGTGT ATAAATAAATGAACGGAGAATTTTCGAG	
Ch11_SEDp-L	TGTAAAACAGGTATTGGCTGCTTCATAGT ACACCCAATTGATTGGATATAGAAAATTA ACGTAAGGCAGTATC	Amplify homology repair template DNA (*SED1$_P$-A.a.BGLI-DIT1$_T$*) with Chromosome 11 target homology
CH11_DITt-R	GCAACTCTGAAATGTCAAAACGGTCGTGT ATAAATAAATGTTACTCCGCAACGCTTTT CTG	
Confirmation of gene integrations		
EGR-Rev	ATCTGGATTAGTAACTTGAGACAAAGCAG	Confirm *T.r.EGII* integration in transformed strains
ENO1-L	GTAACATCTCTCTTGTAATCCCTATTCCTT CTAGC	
CBHIR-Rev	TGTTGAGAGAAGTCGTCGGTGTCAC	Confirm *T.e.CBHI* integration in transformed strains
ENO1-L	GTAACATCTCTCTTGTAATCCCTATTCCTT CTAGC	
CLCBHII-L	AGTCTTAATTAAACAATGGCCAAGAAGTT GTTC	Confirm *C.l.CBHII* integration in transformed strains
CLCBHII-R	AGTCGGCGCGCCTTAGAATGGTG	
BGLR-Rev	GGTTCATCATGTAAGAGTTTTCGC	

ENO1-L	GTAACATCTCTCTTGTAATCCCTATTCCTT CTAGC	Confirm *ENO1$_P$-S.f.BGLI-ENO1$_T$* integration in transformed strains
BGLR-Rev PGK-L	GGTTCATCATGTAAGAGTTTTCGC CTAATTCGTAGTTTTTCAAGTTCTTAGATG C	Confirm *PGK1$_P$-S.f.BGLI-PGK1$_T$* integration in transformed strains
Ch11_SEDp-L	TGTAAAACAGGTATTGGCTGCTTCATAGT ACACCCAATTGATTGGATATAGAAAATTA ACGTAAGGCAGTATC	Confirm *A.a.BGLI* integration in transformed strains
CH11_DITt-R	GCAACTCTGAAATGTCAAAACGGTCGTGT ATAAATAAATGTTACTCCGCAACGCTTTT CTG	
Confirmation of chromosomal integrations at correct loci		
Ch.10 Check-L *T.r.EGII*-R	GCAGTTATCTCTGTGTCCAGATCC GTACGGCGCGCCTTATAACTTTCTAGCCA AACATG AAGAAAG	Confirm *T.r.EGII* integration at Chromosome 10 target site/loci
DeltaCheck-L ENO1-R	CTGTTGGAATAAAAATCCACTATCGTC GCAACCCTATATAGAATCATAAAACATTC GTGA	Confirm *T.e.CBHI* integration at delta (Δ) target sites/loci
DeltaCheck-L CLCBHII-R	CTGTTGGAATAAAAATCCACTATCGTC AGTCTTAATTAAACAATGGCCAAGAAGTT GTTC	Confirm *C.l.CBHII* integration at delta (Δ) target sites/loci
Ch.11Check-L ENO1-R	GCCTTCGATTTGACACATCTCTAAGC GCAACCCTATATAGAATCATAAAACATTC GTGA	Confirm *S.f.BGLI* integration at Chromosome 11 target site/loci
Ch.11Check-L PGK-R	GCCTTCGATTTGACACATCTCTAAGC ACTATTATTTTAGCGTAAAGGATGGGG	Confirm *S.f.BGLI* integration at Chromosome 11 target site/loci
Ch.11Check-L CH11_DITt-R	GCCTTCGATTTGACACATCTCTAAGC GCAACTCTGAAATGTCAAAACGGTCGTGT ATAAATAAATGTTACTCCGCAACGCTTTT CTG	Confirm *A.a.BGLI* integration at Chromosome 11 target site/loci

2.2. Microbial strain cultivations

All chemicals and media components used were of laboratory grade and purchased from Sigma/Merck (St. Louis, MO, USA), unless otherwise stated. Microbial yeast strains, as listed in Table 2.1, were streaked from 15% (v/v) glycerol stocks stored at -80°C onto YPD agar (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) medium supplemented with 100 µg.mL^{-1} CloNAT (Werner Bioagents, Cospeda, Germany), and/or 200 µg.mL^{-1} Geneticin G418 (Invitrogen, Waltham, MA, USA) as required, followed by incubation at 30°C for 48-72 hours. Following cultivation on agar media, YPD broth supplemented with 100 µg.mL^{-1} CloNAT and/or 200 µg.mL^{-1} Geneticin G418, or without selection, as required, was inoculated with the streaked yeast cultures for incubation at 30°C for 72 hours on an orbital shaker at 180 rpm.

Plasmids, as listed in Table 2.2, were propagated from *Escherichia coli* DH5*α* 40% (v/v) glycerol stocks stored at -80°C by streaking out on LB agar (0.5% yeast extract, 1% tryptone, 1% NaCl, and 2% agar) supplemented with 100 μg.mL^{-1} ampicillin (Roche; Basel, Switzerland), followed by overnight incubation at 37°C. To prepare cultures for plasmid DNA isolation, single colonies were inoculated in liquid LB media supplemented with 100 μg.mL^{-1} ampicillin, followed by overnight incubation at 37°C on a rotary wheel.

2.3. Plasmid DNA isolation, restriction digestion, and PCR amplification

Plasmid DNA isolation from *E. coli* DH5*α* cultures was performed using the cetyl trimethyl ammonium bromide (CTAB) method (Del Sal *et al.*, 1988). To verify the sizes of each respective gene cassette and/or CRISPR gRNA sequences, isolated plasmid DNA was subjected to restriction digestion at 37°C with *PacI* and *AscI* and/or *EcoR1* (Thermo Fisher Scientific Waltham, MA, USA), respectively, followed by separation on a 1% (w/v) agarose gel. Following confirmation (not shown), gene cassettes (shown in Table 2.2) were PCR amplified with specific primers (shown in Table 2.3) with cycling conditions shown in Table 2.4.

Table 2.4: PCR cycling conditions to amplify gene cassettes used as homology repair templates

Cassettes	Initial denaturation	31 cycles of	Final Extension; Hold
T.r.EGII, *T.e.CBHI*, *C.l.CBHII*	95°C for 5 min	Denaturation (95°C for 30 sec) Annealing (58°C for 30 sec) Elongation (72°C for 2 min, 45 sec)	72°C for 7 min; 4°C (∞)
S.f.BGLI	95°C for 2 min	Denaturation (95°C for 30 sec) Annealing (50°C for 30 sec) Elongation (72°C for 3 min, 30 sec)	72°C for 7 min; 4°C (∞)
A.a.BGLI	95°C for 5 min	Denaturation (95°C for 30 sec) Annealing (60°C for 30 sec) Elongation (72°C for 5 min, 20 sec)	72°C for 7 min; 4°C (∞)

To purify the resolved gene cassette PCR products and CRISPR plasmid DNA from the agarose gel, DNA bands were extracted using the Freeze-and-Squeeze method (Thuring *et al.*, 1975) and further purified with the use of phenol:chloroform:isoamyl alcohol (PCI; 25:24:1). Following purification, isolated DNA was subjected to dialysis against purified water and a 0.025 μm MCE membrane filter (Merck Millipore; Burlington, MA, USA) and subsequently

to quantitative spectrophotometric analysis (NanoDrop2000, ThermoScientific) to determine the DNA concentration for subsequent use in transformation.

2.4. Electro-transformation of yeast strains with CRISPR plasmids and target genes and screening of putative positive transformants

Transformation of yeast strains with homology repair template DNA (*EGII*, *CBHI*, *CBHII* or *BGLI* gene cassettes), the pCas9-NAT plasmid, and the CRISPR plasmid targeting a specific intergenic region on chromosomes 10 or 11, or delta sequences throughout the yeast genome, were conducted as described by Cho and co-workers (1999) with minor adaptations to permeabilization of yeast cells to allow for improved transformation efficiencies (Moriguchi *et al.*, 2016). Briefly, harvested cells were washed with sterile distilled water, followed by resuspension in LiOAc/TE (0.1 M LiOAc, 10 mM Tris-HCl pH 8.0, and 1 mM EDTA). Resuspended cells were then incubated at 30°C for 45 minutes with shaking, prior to the incubation of cells for 15 minutes with added 1 M DTT. The mixture was then centrifuged, and cells washed with sterile distilled water, followed by resuspension in electroporation buffer (1 M sorbitol, 20 mM HEPES). Competent cells were transformed with ~10 µg template DNA and ~1 µg CRISPR plasmid DNA under standard electroporation conditions (1.4 kV, 200 ohms, 25 µF) using a micropulser (BioRad; Hercules, CA, USA). Following electroporation, cells were suspended in 1 ml YPD broth media supplemented with 1 M sorbitol, followed by overnight incubation at 30°C on an orbital shaker at 180 rpm. The transformation mixture was then plated on YPD agar medium supplemented with CloNAT (100 µg.mL^{-1}) and/or Geneticin G418 (200 µg.mL^{-1}) and incubated for 48-72 hours at 30°C.

Putative positive transformants obtained from transformation plates were then streaked on YPD media supplemented with CloNAT (100 µg.mL^{-1}) and/or Geneticin G418 (200 µg.mL^{-1}), followed by incubation at 30°C for 24-48 hours, prior to inoculating overnight YPD cultures for quick yeast DNA extractions, as described by Hoffman and Winston (1987). Isolated yeast DNA were then used as DNA templates to confirm the presence of integrated cellulase genes at the correct target intergenic regions with PCR analyses, using specific primers as listed in Table 2.3.

2.5. Enzyme activity assays

To identify transformants with high secretory profiles for all heterologous enzymes, preliminary enzyme activity assays were conducted, by inoculating 5-10 PCR confirmed

positive transformants into 5 mL YPD liquid media for 48 hours at 30°C on an orbital shaker at 180 rpm. Superior secretors for each respective isolate were selected based on high activity profiles for all recombinant genes, with the main determinant being for CBHI activity. Confirmed isolates were then cultivated in biological triplicates in 10 mL YP media supplemented with 2% glucose, under various fermentation-related conditions, namely, (1) 30°C, (2) 37°C, and (3) 30°C in the presence of 3 g/L acetic acid, respectively, for 72 hours with shaking at 180 rpm.

Following cultivation, EG activity was quantitated using the dinitrosalicylic acid (DNS) method as described by Bailey and co-workers (1992), using sodium acetate (50 mM, pH 5.0) as buffer and carboxylmethyl cellulose (CMC, 1% w/v) as substrate. Briefly, cell free supernatants and the CMC substrate were incubated at 50°C for 60 minutes, followed by inhibiting the enzyme reaction with DNS. The volumetric values obtained were normalised with the dry cell weight (DCW) of each respective isolate included in the assay in g/L (Meinander *et al.*, 1996). The enzyme activities obtained for the respective isolates were expressed as units/g DCW, where one unit (U) was equivalent to the amount of enzyme required to release 1 µmol of reducing sugar or equivalent per minute. A DNS standard curve (Appendix A, Figure S3) in the range of 2-10 g/L glucose was used to determine enzyme activity.

CBH activity was quantitated using soluble fluorescent methylumberiferyl-β-lactopyranoside (MULac, Sigma) as substrate, as described by Ilmén and co-workers (2011). Briefly, cell free supernatants were incubated at 37°C for 20 minutes, followed by inhibiting the enzyme reaction with 1 M sodium carbonate prior to measuring fluorescence (excitation wavelength = M 355nm, emission wavelength = 460 nm) using a FLUOstar Omega Microplate Reader (BMG LABTECH; Ortenberg, Germany). The amount of fluorescence emitted by each sample was compared against methylumbelliferone MU standard curve (Appendix A, Figure S4) set in the range of 0.63-20 µM, and enzyme activity was expressed as units/g DCW.

For BGL activity, recombinant and wild-type isolates were assayed with ρ-nitrophenyl-β-D-glucopyranoside (pNPG), as described by Van Zyl and co-workers (2014) with slight adaptations. Briefly, whole cell cultures were assayed at 50°C for 30 minutes, prior to inhibiting the enzyme reaction with 1 M sodium carbonate. Cell cultures were then subjected to centrifugation at 3000 rpm for 2 minutes, after which 100 µL of cell free supernatants were used for spectrophotometric measurements at 400 nm. Obtained volumetric values were then

compared against a pNP standard curve in the range of 0.075-1.25 mM (Appendix A, Figure S5), and enzyme activity were expressed as units/g DCW.

2.6. Avicel hydrolysis

To evaluate the percentage of Avicel converted by secreted cellulolytic enzymes, a substrate mixture containing 2% w/v Avicel PH101 (Fisher Scientific; Hampton, NH, USA), sodium azide (0.02%), and sodium acetate (50 mM, pH 5.0) were prepared by continuous mixing to ensure homogeneity, as described by Chetty and co-workers (2022) with slight modifications. For isolates with low BGL secretory profiles, additional BGL (Novozyme-188, Sigma) was added to ensure sufficient liberation of glucose. Into a deep 96-well plate, substrate mixture and yeast culture supernatant were added at a 1:1 ratio, followed by incubation at 35°C with shaking at 1000 rpm in a Heidolph Titramax 1000 microplate shaker/incubator. Samples were taken at 0, 24, and 48 hours, to measure the amount of glucose liberated by enzymatic hydrolysis, using an adapted DNS assay procedure (Den Haan *et al.*, 2013).

2.7. Strain robustness against bioethanol-related production and secretion stresses

Transformed and background strain isolates were cultivated at 30°C for 48 hours in YPD liquid media, after which cell densities of cultures were measured at OD_{600nm} for standardisation, using YPD as diluent to a final volume of 1 mL. Ten-fold serial dilutions (first dilution, OD_{600nm} = 1.0) were then performed for each respective culture, followed by spotting 3 µL on YPD agar media supplemented with the appropriate inhibitory component. The inhibitors screened for that were specific to bioethanol production included: ethanol (8% w/v) and NaCl (1.2 M). To evaluate the response to heat stress induced by the different steps within the production process, isolates were also cultivated on YPD agar media at 30°C and 40°C. In addition, isolates were also evaluated for their robustness against acetic acid (5 g/L) stress.

To evaluate tolerance towards ER and cell wall stressors, strains were cultivated on YPD solid media supplemented with Tunicamycin (1 $\mu g.mL^{-1}$) and Congo Red (600 $\mu g.mL^{-1}$), respectively. Both plate assays were conducted in ten-fold serial dilutions, followed by incubation at 30°C for 48 hours. Strains were compared with regards to their sensitivity towards various inhibitors or conditions based on their growth at various dilutions.

2.8. Growth curve analyses

Cell growth assays for wild-type and recombinant isolates were conducted as described by Chetty and co-workers (2022). Briefly, cell cultures were inoculated in 5 mL YPD liquid media and incubated overnight at 30°C with shaking (180 rpm). Cultures were then inoculated to an OD_{600nm}=0.5 in flasks containing 10 mL YPD prior to incubation under the same conditions. Samples were taken every 2 hours until the stationary phase was reached. Appropriate dilutions were made at each sampling time, and OD_{600nm} readings were taken using a FLUOstar Omega Microplate Reader (BMG LABTECH). Growth analysis was conducted in biological triplicates, and the OD values obtained were given as the average of repeats with their respective standard deviations.

2.9. Fermentation of Avicel

To evaluate ethanol production of isolates on microcrystalline cellulose, isolates were inoculated in YP media supplemented with Avicel (2% w/v), as described by Chetty and co-workers (2022) with slight modifications. Briefly, isolates were pre-cultured in 50 mL YPD liquid media for 96 hours at 37°C with shaking at 180 rpm. Rubber-stoppered glass bottles (Lasec, Cape Town, South Africa) containing 10 mL double strength YP media (20 g/L yeast extract, 40 g/L peptone) supplemented with 40 g/L Avicel were inoculated with 10 mL pre-cultures, in biological triplicates (to achieve a concentration of 20 g/L Avicel) followed by incubation at 30°C for 120 hours. Oxygen-limited conditions were maintained by piercing rubber stoppers with 0.8x25 mm syringe filters plugged with cotton wool, to act as outlets for CO_2. Sample volumes of 1 mL were taken at 0, 72, and 120-hours during fermentation, and subsequently stored at -20°C until further analysis.

2.10. High Performance Liquid Chromatography (HPLC) analysis

Samples collected during fermentation were subjected to centrifugation at 13 000 rpm for 10 minutes, prior to transferring the cell free supernatant to a clean 1.5 mL Eppendorf tube. To acidify samples, 10% (v/v) sulfuric acid (H_2SO_4) solution was added to each sample, followed by a brief vortex to mix. Samples were then filtered through a 0.22 µm filter into 2 mL HPLC vials. Ethanol, cellobiose, acetic acid, glucose, and glycerol concentrations were determined in each sample by HPLC equipped with a BioRad guard (part # 125-0129) and refractive index (RI) detector. Compound separation was achieved on a BioRad Aminex HPX-87H (part # 125-0140) 7.8x300 mm column at a temperature of 65°C, with 5 mM H_2SO_4 as mobile phase at a

flow rate of 0.7 mL/min. Values obtained for each respective compound were presented as the mean of triplicates, in g/L, with their standard deviations.

2.11. Statistical analysis

Significant differences between enzyme activities, growth data and/or metabolite concentrations attained were investigated using two-tailed T-tests, assuming unequal variance. A *p*-value lower than 0.05 was deemed significant.

CHAPTER 3: RESULTS AND DISCUSSION

3.1. Strain construction and confirmations

Heterologous protein production and secretion of cellulolytic enzymes in *S. cerevisiae* is affected by various fermentation-associated stresses in bioethanol production (Lamour *et al.*, 2019). The growth and viability of individual strain isolates are affected by temperatures higher than their optimal growth temperature, as well as the presence of inhibitory compounds such as acetic acid, high salt and ethanol concentrations, as well as the presence of inhibitory compounds commonly present in lignocellulosic hydrolysates. However, tolerance towards these variables is strain-dependant. Hence, this study aimed to evaluate the secretory profiles of heterologous cellulases using promising natural isolates that were previously identified (Davison *et al.*, 2016), in comparison with the reference industrial strain, MH1000. To create yeast strains expressing a core set of cellulases in these robust strain backgrounds, we initially transformed natural and reference *S. cerevisiae* isolates with pCas9-NAT, and with each respective gRNA plasmid and homology repair cassette, corresponding to *T.r.EGII*, *S.f.BGLI*, and *T.e.CBHI*, in successive rounds of transformation. Through the CRISPR/Cas9 system, these gene cassettes could be integrated into targeted intergenic regions on Chromosome 10 or 11, or in the repeated δ-sequences, as previously shown (Jacob, 2022; Kruger and Den Haan, 2022). For each successive round of transformation, a homology repair cassette with its respective gRNA plasmid was transformed into the individual isolates (Tables 2.1 and 2.2; Figure S1). Once successful integration of the cellulase-encoding gene was confirmed, the gRNA plasmid was cured from the strain with successive rounds of non-selective sub-culturing, prior to the next round of transformation. To confirm whether the isolates were successfully transformed, PCR amplification was performed on putative positive transformants (Figure 3.1).

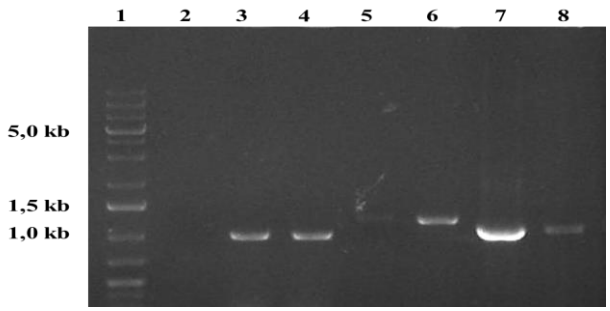

Figure 3.1.: Confirmation of *T.r.EGII, T.e.CBHI,* and *S.f.BGLI* integration in the natural *Saccharomyces cerevisiae* YI59 isolate to create the strain YI59_ECBE. Lane 1: 1 kb DNA ladder for Safe Stains (NEB); Lane 2: YI59_WT; Lane 3: Y294_EGII; Lane 4: Y159_WT + *T.r.EGII*; Lane 5: Y294_CBHI; Lane 6: YI59_WT + *T.e.CBHI*; Lane 7: Y294_BGLI; Lane 8: YI59_WT + *S.f.BGLI*.

As illustrated in Figure 3.1, successful integration of *T.r.EGII*, *T.e.CBHI*, and *S.f.BGLI* into *S. cerevisiae* YI59 was confirmed by performing colony PCR. Successful integration for *T.r.EGII* was indicated by the presence of a band of approximately 1.0 kb (lane 4), when using primer sequences as listed in Table 2.3. *S.f.BGLI* yielded a fragment of approximately 1.0 kb (lane 8), confirming successful integration. Integration of *T.e.CBHI* was indicated by a band corresponding to approximately 1.2 kb (lane 6). Successful integration for all genes as listed in Table 2.1 was achieved in all the natural and industrial strain isolates (Figure S6). We could therefore conclude that CRISPR-based transformation of the *S. cerevisiae* isolates was successful to allow for integration of the relevant heterologous cellulase-encoding genes.

3.2. Enzyme activity assays

To evaluate the secretory profiles of the heterologous cellulases in laboratory conditions and how this might change in processing conditions, strains were cultivated in the optimal yeast growth temperature (30°C) (Munna *et al.,* 2015) and at a higher than optimal temperature (37°C). In addition, the effect of acetic acid on the cell growth and heterologous enzyme secretion was also evaluated at 30°C. All strains were cultivated for 72 hours under the beforementioned conditions prior to enzyme activity measurements, as described in Chapter 2. It should also be noted that the wild-type strain variants were all evaluated for their potential to secrete cellulases and indicated no significant measurable activity for any of the cellulases tested (shown in Appendix A, Figure S2). Therefore, negative controls were omitted in most of the assay results shown.

3.2.1. β-glucosidase (BGL) activity

BGL catalyses the hydrolysis of cellobiose and some cello-oligosaccharides liberated by action of cellobiohydrolases, to yield glucose monomers that can be utilised by fermenting microorganisms (Mohsin *et al.,* 2019). Therefore, to ensure high cellulose hydrolysis efficiency, optimal BGL activity is required. Initial assays of our transformants resulted in low BGL activity. We therefore attempted to improve BGL activity by expressing the *S.f.BGLI* gene under a different constitutive promoter ($PGK1_P$).

As illustrated in Figure 3.2, strain constructs containing the *S.f.BGLI* gene cassette under transcriptional control of either the *ENO1* promoter and terminator (_ECBE), or the *PGK1* promoter and terminator (_ECBP), both yielded BGL activity levels that were relatively low, ranging between 0.46-3.36 U/g DCW. However, differences were observed between transformants expressing *S.f.BGLI* under control of the different regulatory sequences, despite

the genes being targeted to the same chromosomal locus, as indicated in Table 2.1. By cultivating transformed isolates under different 2G fermentation-associated conditions (i.e., optimal cultivation temperature (30°C), high temperature (37°C), and/or in the presence of 3 g/L acetic acid), superior secretory capacities were observed for YI13_ECBE, FIN1_ECBE, and YI59_ECBP. As shown in Figure 3.2A, YI13_ECBE and FIN1_ECBE exhibited superior secretory capacity under high temperature and/or in the presence of 3 g/L acetic acid, correlating to more than 4.0- and 1.5-fold higher BGL activity than the transformed reference strain, MH1000_ECBE, respectively. However, under optimal cultivation conditions (i.e., 30°C), MH1000_ECBE exhibited the highest extracellular BGL activity (1.14 U/g DCW) compared to the transformed natural isolates YI13_ECBE, FIN1_ECBE, and YI59_ECBE. Similarly, differences were also observed between transformed natural isolates and compared to the reference strain for BGL expressed with the *PGK1* promoter and terminator sequences (Figure 3.2B). While YI59_ECBP displayed similar BGL activity levels as MH1000_ECBP under high temperature conditions (1.52 U/g DCW and 1.36 U/g DCW, respectively), the strains' secretory capacity for BGL under optimal cultivation conditions (2.62 U/g DCW) and in the presence of 3 g/L acetic acid (3.36 U/g DCW) was superior to that of FIN1_ECBP, YI13_ECBP and MH1000_ECBP, under the same beforementioned conditions.

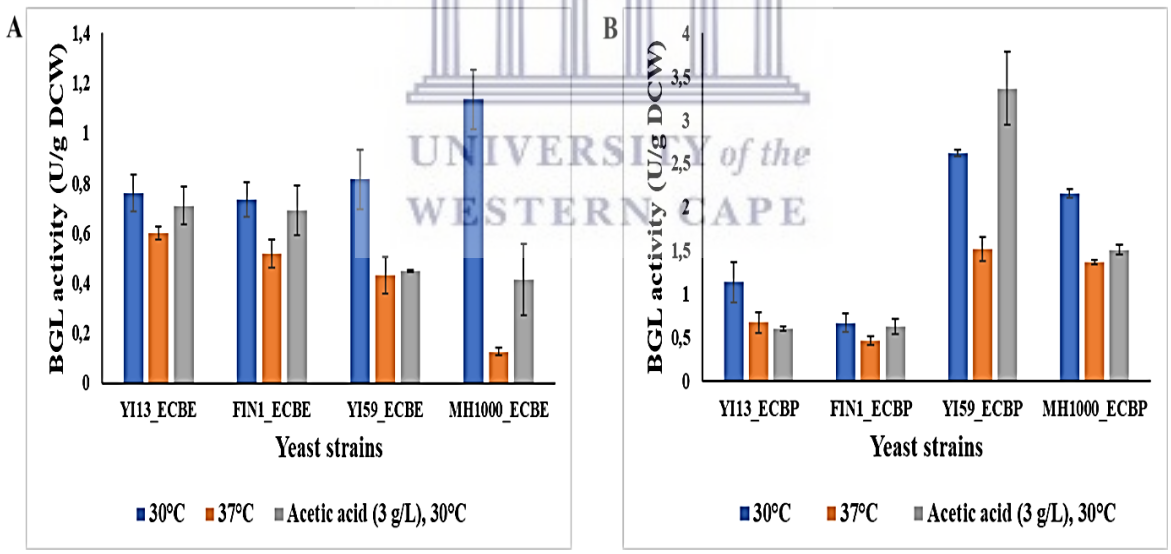

Figure 3.2: Beta (*β*)-glucosidase (BGL) activity assayed in various cultivation conditions based on the *ρ*-nitrophenyl-*β*-D-glucopyranoside (pNPG) method. Yeast strains were cultivated in 10 ml YP media supplemented with 2% (v/v) glucose for 72 hours at 30°C (blue bars), 37°C (orange bars), and in the presence of 3 g/L acetic acid (grey bars). Two variations of each strain isolate were plotted as (A) ECBE and (B) as ECBP, with the distinction based on the regulatory sequence of the *S.f.BGLI* gene cassette. Volumetric values (U/L) obtained were standardised with the dry cell weight (DCW) of each respective isolate in g/L. Data bars represent the average of three biological repeats per strain, with error bars representing the standard deviation.

Based on the differences observed amongst transformants, and in comparison with the respective reference strains, it was observed that the regulatory sequence(s) used for *S.f.BGLI* expression played a critical role in the enzyme activity levels achieved by transformants. Although strong endogenous constitutive promoters (i.e., *PGK1*$_P$ and *ENO1*$_P$) were used for expression of the heterologous hydrolase, several factors may have influenced the transcriptional regulatory mechanism of the gene expression (Peng *et al.,* 2015). For instance, the cultivation conditions may have caused a decrease in the expression of the BGL enzyme as a result of glucose depletion, which may have blocked the transcription of the gene (Xiong *et al.,* 2018). Furthermore, optimal gene expression is dependent on different environmental stimuli, hence operation of these promoters under variable stressful conditions may have caused changes in the promoters' dynamic range and intensity (Li *et al.,* 2022), which could have resulted in decreased *S.f.BGLI* transcript levels and subsequently low extracellular enzyme activity. In addition, the rate of transcription of native promoters is tuned according to the natural setting of the host strain (Chen *et al.,* 2018), which suggested that the superior secretory profile obtained for YI59_ECBP corresponded to the host strain having a diverse genetic background (Davison *et al.,* 2016) which could have allowed for increased gene expression and secretion of the BGL enzyme.

While the use of *PGK1*$_P$ improved BGL activity in most strains and conditions tested, it was still found to be comparatively low for recombinant cellulase-producing yeast strains (Chetty *et al.,* 2022; Den Haan *et al.,* 2021). Expression of an alternative BGL was thus explored. Selection of the BGL-encoding gene was based on research conducted by Inokuma and co-workers (2014, 2016, and 2021), where the researchers managed to successfully enhance expression of a cell-surface displayed BGL by optimising promoter and/or terminator sequences to allow for a 17.0-fold increase in enzyme activity when compared to conventional constitutive promoters, such as *PGK1*$_P$ and *TDH3*$_P$. In essence, Inokuma and co-workers showed that by expressing *A. aculeatus* β-glucosidase 1 (*A.a.BGLI*) under transcriptional control of the *S. cerevisiae SED1*-derived promoter (*SED1*$_P$) and *S. cerevisiae DIT1*-derived terminator (*DIT1*$_T$), a significant increase in activity could be achieved. In this study, we therefore constructed additional strains by integrating the newly acquired BGL gene cassette, after receiving it from our collaborator Prof. Tomohisa Hasunuma at Kobe University, Japan (Table 2.1).

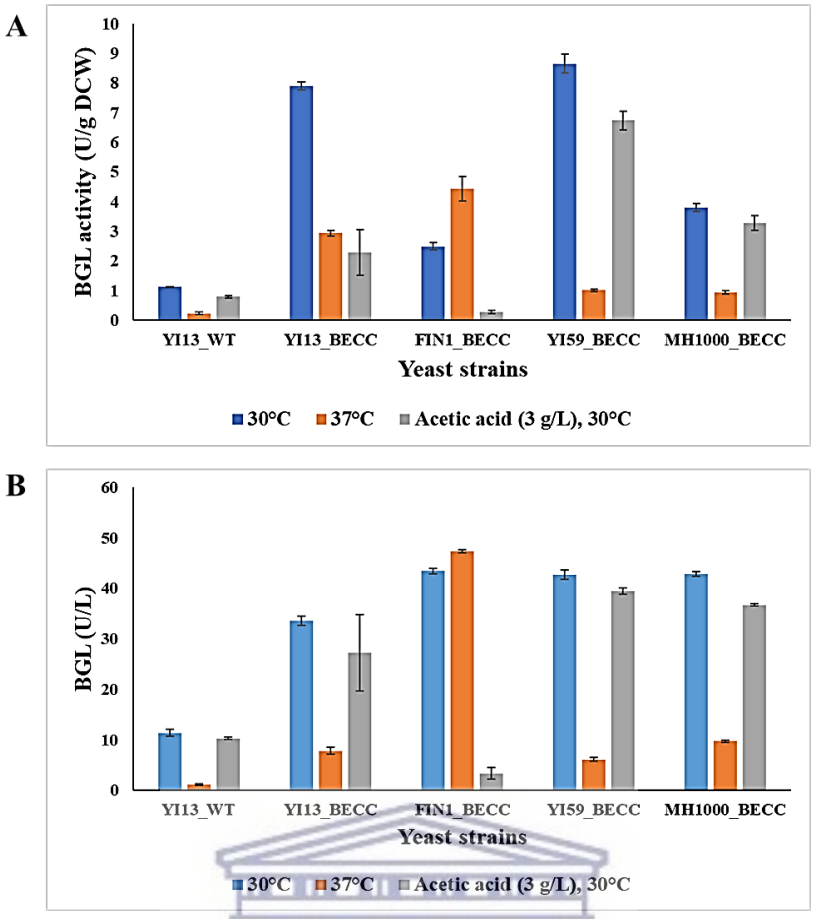

Figure 3.3: Beta (*β*)-glucosidase (BGL) activity of additional strains assayed in various cultivation conditions based on the *ρ*-nitrophenyl-*β*-D-glucopyranoside (pNPG) method. To evaluate the total enzyme produced by the transformed isolates, the (A) specific activity and (B) volumetric activity values were compared. Yeast strains were cultivated in YP media supplemented with 2% (v/v) glucose for 72 hours at 30°C (blue bars), 37°C (orange bars), and in the presence of 3 g/L acetic acid (grey bars). Volumetric values (U/L) obtained were standardised as required with the dry cell weight (DCW) of each respective isolate in g/L, and specific enzyme activities are thus expressed as units/g DCW. Data bars represent the average of three biological repeats per strain, with error bars representing the mean ± standard deviation.

As illustrated in Figure 3.3A, significant differences could be observed among transformed natural isolates, and the reference strain, MH1000_BECC, under all the cultivation conditions. Superior secretory capacity for YI59_BECC was noted at 30°C (8.65 U/g DCW) and in the presence of 3 g/L acetic acid (6.74 U/g DCW), which correlated to a 2.28- and 2.06-fold higher enzyme activity compared to the MH1000_BECC strain. Conversely, FIN1_BECC yielded superior secretory capacity at higher cultivation temperature (4.43 U/g DCW), allowing for a 4.82-fold higher enzyme activity compared to the reference strain. This latter result agreed with the result obtained by Davison and co-workers (2016), who reported that this natural isolate displayed an increased tolerance to higher temperatures and suggested that heterologous secretion was dependant on the hosts' genetic background.

The high specific BGL activity levels achieved for YI59_BECC and FIN1_BECC illustrated in Figure 3.3A might suggest that a rapid increase in cell numbers was achieved to allow for the efficient functioning of the internal secretion pathway and other macromolecule structures within the natural isolate cells (Munna *et al.*, 2015); however, this seemed to not be the case. As illustrated in Figure 3.3B, the volumetric activity levels under optimal conditions obtained for FIN1_BECC, YI59_BECC, and MH1000_BECC displayed no significant differences, with the only exception being YI13_BECC which had slighly lower volumetric activity. This means that a lower cell number was required for YI59_BECC to achieve similar volumetric activity as FIN1_BECC and MH1000_BECC, showing the superior ability of the former to secrete the BGL in these conditions. Similarly, YI13_BECC yielded specific activity levels similar to that of YI59_BECC, despite its lower volumetric activity levels. Thus, it can clearly be seen that YI59_BECC and YI13_BECC had superior secretory capacity for *A.a.BGLI* compared to the other strains. Due to the superior activity achieved with the $SED1_{P}$-*A.a.BGLI*-$DIT1_{T}$ cassette, we continued only with strains carrying this gene along with the other cellulases (i.e., $ENO1_{P}$-*T.r.EGII*-$ENO1_{T}$ and $ENO1_{P}$-*T.e.CBHI*-$ENO1_{T}$). In addition, these strains were also equipped with an additional cellobiohydrolase enzyme, namely *C.l.CBHII*, which provided transformed strains with a core cellulase complex, necessary for efficient lignocellulose hydrolysis (Appendix A, Figure S1-C).

3.2.2. Cellobiohydrolase (CBH) activity

While high BGL activity is necessary for an optimal hydrolysis of lignocellulosic feedstocks and to ensure that CBH inhibition is avoided, optimal hydrolysis of crystalline feedstocks requires superior activity of CBHs (Taylor *et al.*, 2018). Cultivation of transformed natural isolates and the reference industrial strain under all assay conditions (i.e., 30°C, 37°C, and in the presence of 3 g/L acetic acid), yielded relatively low secretory activity for *T.e.*CBHI, as illustrated in Figure 3.4A. It should be noted that the assay used can only determine CBHI activity and that the *C.l.*CHBII activity of these strains cannot be directly measured. Although the *T.e.*CBHI gene cassette was integrated in strain isolates to be controlled under the same regulatory mechanism ($ENO1_{P/T}$), variation in the extracellular activity profiles existed among the isolates ranging from 0.002-0.259 U/g DCW. YI13_BECC yielded the highest CBH activity at 37°C (0.259 U/g DCW), followed by 30°C (0.046 U/g DCW), and in the presence of 3 g/L acetic acid (0.008 U/g DCW). Moderate enzyme activity was achieved for FIN1_BECC under optimal conditions and at increased temperature; however, in the presence of 3 g/L acetic acid, its activity was significantly lower (0.002 U/g DCW). In comparison with

the reference MH1000_BECC strain, YI13_BECC yielded 4.18, 51.8, and 2-fold higher extracellular CBHI activity on a per DCW bases under each of the cultivation conditions. This agreed with the study conducted by Davison and co-workers (2016), despite the use of episomal plasmids for expression of the heterologous enzymes in that study.

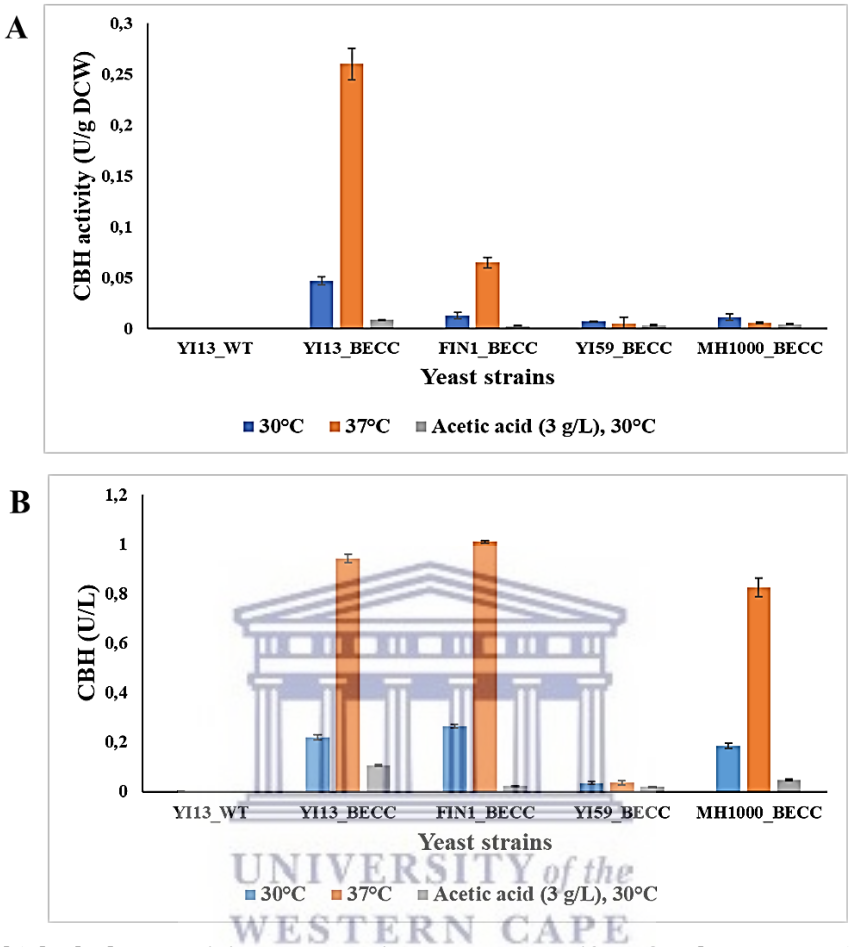

Figure 3.4: Cellobiohydrolase I activity assayed using methylumbelliferyl β-D-lactopyranoside (MULac) as substrate. The (A) specific activity and (B) volumetric activity values were compared. Yeast strains were cultivated in YP media supplemented with 2% (v/v) glucose for 72 hours at 30°C (blue bars), 37°C (orange bars), and in the presence of 3 g/L acetic acid (grey bars). Volumetric values (U/L) were standardised with the dry cell weight (DCW) of each strain in g/L, and specific enzyme activities were thus expressed as units/g DCW. Data bars represent the average of three biological repeats per strain, with error bars representing the mean ± standard deviation.

Variation in the activity profiles was observed between volumetric and specific CBH activities (Figure 3.4). As illustrated in Figure 3.4B, YI13_BECC, FIN1_BECC, and MH1000_BECC displayed high volumetric activity levels at 37°C; however, compared to the specific activity levels of each, it was seen that the higher cell numbers of FIN1_BECC and MH1000_BECC caused the specific activity levels to appear reduced, compared to that of YI13_BECC. This shows that while the FIN1_BECC and MH1000_BECC strains grew relatively well in the high temperature conditions, they produced comparatively less CBH, indicating that YI13_BECC

possessed superior cellobiohydrolase secretory capacity in these conditions. Therefore, we concluded that the YI13_BECC strain had superior secretory capacity for production of CBHI, as well as for BGLI, illustrating the efficient heterologous protein secretory capabilities of this host strain.

3.2.3. Endoglucanase (EG) activity

Variable specific EG activity levels across all cultivations were observed between transformed natural isolates and the reference strain (Figure 3.5). Enzyme activity levels ranged between 1.42-47.23 U/g DCW (Figure 3.5A). YI13_BECC displayed superior secretory capacity under optimal conditions (35.90 U/g DCW) and at higher temperatures (47.23 U/g DCW), followed by YI59_BECC which displayed superior secretory capacity in the presence of 3 g/L acetic acid (28.70 U/g DCW). This corresponded to 2.14-, 4.2-, and 2.2-fold higher enzyme activity levels for each assay condition over the reference strain.

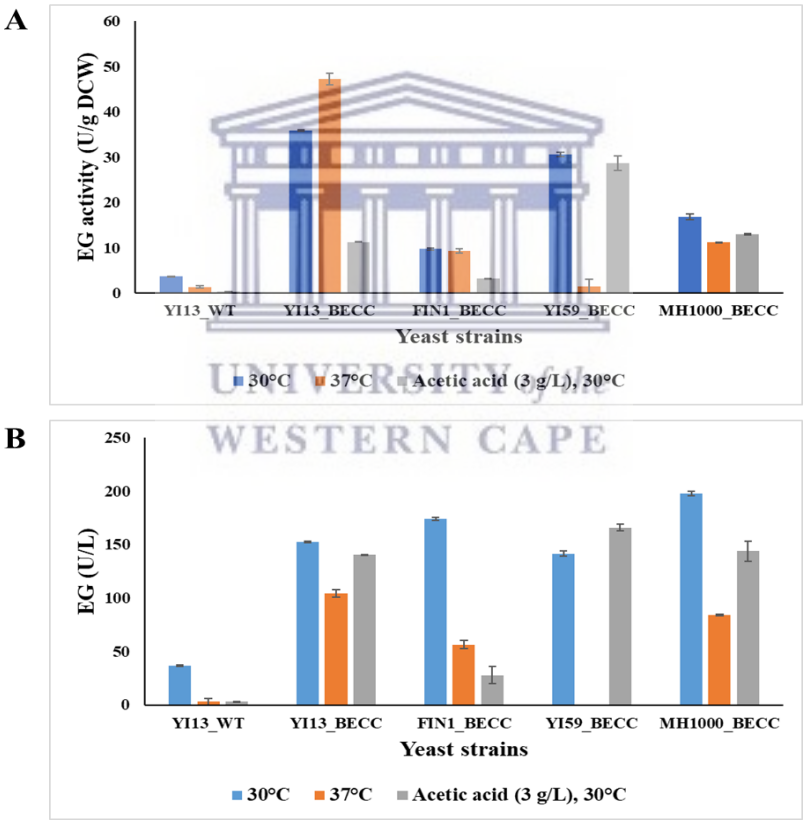

Figure 3.5: Endoglucanase activity assayed with the dinitrosalicylic acid (DNS) method. The (A) specific activity and (B) volumetric activity values were compared. Yeast strains were cultivated in YP media supplemented with 2% (v/v) glucose for 72 hours at 30°C (blue bars), 37°C (orange bars), and in the presence of 3 g/L acetic acid (grey bars). Volumetric values (U/L) obtained were standardised with the dry cell weight (DCW) of each strain in g/L, and specific enzyme activities were thus expressed as units/g DCW. Data bars represent the average of three biological repeats per strain, with error bars representing the mean ± standard deviation.

Compared with Figures 3.3B and 3.4B, a similar pattern was observed for the volumetric EG activity levels obtained for the transformed natural isolates and the reference strain, as shown in Figure 3.5B, under optimal cultivation conditions. However, in the presence of acetic acid, YI59_BECC, YI13_BECC, and MH1000_BECC displayed similar volumetric activity levels, although only YI59_BECC displayed superior specific activity for the EG enzyme. This corresponded with previous observations that lower cell numbers were required for YI59_BECC to have exhibited superior secretory capacity for heterologous protein secretion.

3.3. Avicel hydrolysis

During lignocellulosic bioprocessing, the efficiency of enzymatic hydrolysis is dependent on the synergistic action of the entire cellulase enzyme complex (Hu *et al.*, 2016). Each enzyme should thus be present in an optimal ratio to ensure no accumulation of intermediates occur that may limit and/or inhibit the action of preceding enzymes. However, a major issue during lignocellulosic degradation processes is the accumulation of cellobiose, the intermediate produced by hydrolytic action of CBHs, as a result of weak BGL activity. Accumulated cellobiose causes an inhibitory effect on CBH, which in turn causes inhibition of EGs due to cellodextrin (product of EG action) accumulation (Behera *et al.*, 2017). Therefore, to increase the efficiency of enzymatic hydrolysis of lignocellulosic substrates in industrial practices, additional BGL is required, adding to production costs (Hu *et al.*, 2016). For this reason, Avicel hydrolysis efficiencies were investigated for the transformed natural and industrial isolate(s) to evaluate the efficiency of enzymatic hydrolysis on a microcrystalline substrate, to determine whether the secreted cellulases functioned optimally.

As illustrated in Figure 3.6, significant differences were observed between natural isolates, in comparison with the reference strain. Under optimal cultivation conditions, an Avicel conversion efficacy in the range of 0.26-1.46 % per g DCW was observed. As expected, the conversion efficiency for YI13_BECC was superior to that of the other transformed natural isolates (1.08 % per g DCW), due to superior heterologous cellulase production under these conditions. Somewhat surprisingly, compared to the reference strain MH1000_BECC, a 1.35-fold lower conversion efficiency was obtained. The better conversion efficiency obtained for MH1000_BECC may be due to strain background, as domestication events may have improved some phenotypic characteristics of the isolate (Mukherjee *et al.*, 2014). In addition, the cellulases secreted by the MH1000_BECC isolate may have been present in optimal ratios to allow for a higher conversion efficiency; however, this cannot be confirmed as no gene copy

number or transcriptomic analysis was performed and the amount of CBHII activity could not be measured directly.

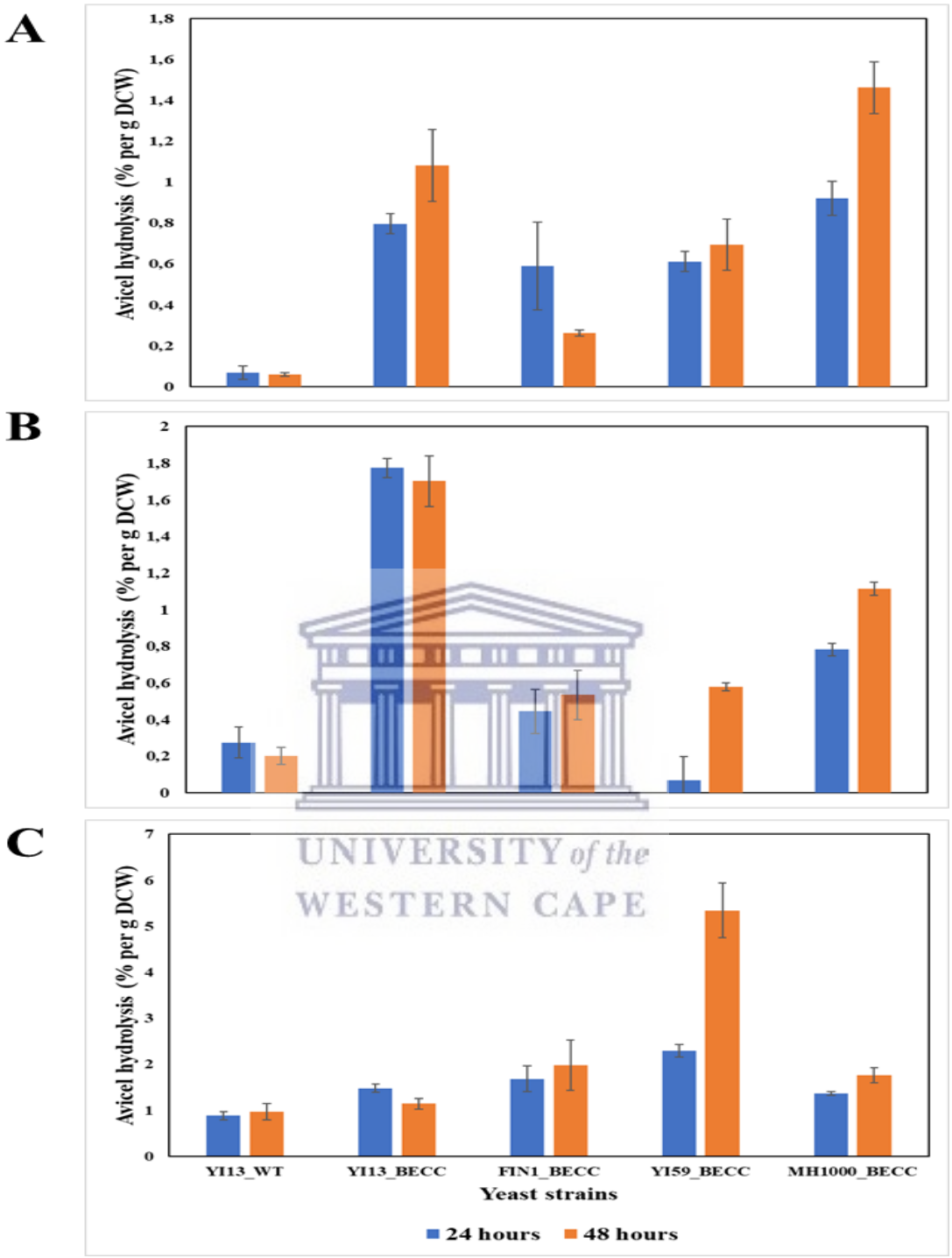

Figure 3.6: Avicel hydrolysis of transformed natural and industrial strain isolates in various growth conditions. Yeast strains were cultivated in YP media supplemented with 2% (v/v) glucose for 72 hours at (A) 30°C, (B) 37°C, and (C) in the presence of 3 g/L acetic acid. Cell-free supernatants were inoculated at 1:1 in and Avicel-mixture containing no additional beta (β)-glucosidase (BGL), followed by incubation at 35°C with shaking at 1000 rpm. Avicel hydrolysis (%) was evaluated based on residual sugars determination and standardised with dry cell weight. Data bars represent the average of three biological repeats per strain, with error bars representing the mean ± standard deviation.

At elevated temperature, a conversion efficiency ranging between 0.53-1.77 % per g DCW was obtained, as shown in Figure 3.6B. YI13_BECC, the superior secretor for cellulases at elevated temperatures, displayed superior conversion efficiency as expected, corresponding to a 1.59-fold higher efficacy compared to the reference MH1000_BECC strain. Interestingly, MH1000_BECC exhibited moderate conversion efficiency (1.93- and 2.09-fold higher than YI59_BECC and FIN1_BECC, respectively). This was interesting as MH1000_BECC exhibited relatively low CBHI and BGLI activity levels in these conditions. The higher conversion efficiencies observed for this strain might suggest that optimal expression of the CBHII enzyme along with the other cellulases was achieved in this strain background.

Another interesting result that arose from the Avicel conversion assays was the high conversion efficiencies observed for cellulases produced under acetic acid stress. As the wild-type negative control also had higher values in these conditions, we assumed that acetic acid carried over from the cultivation conditions affected the assay. Even so, the comparative levels of conversion were significantly higher in these conditions. As shown in Figure 3.6C, conversion efficiencies ranged between 1.14-5.34 % per g DCW, with YI59_BECC displaying the highest conversion efficiency (5.34% per g DCW), corresponding to a 3.04-fold higher efficiency than MH1000_BECC. This was expected, as YI59_BECC was the superior secretor for the entire cellulase enzyme complex under acetic acid stress (Figures 3.3 – 3.5). Interestingly, FIN1_BECC displayed a conversion efficiency similar to that of MH1000_BECC, despite displaying relatively low enzyme activity levels for the individual cellulases in this cultivation condidtion.

3.4. Strain robustness evaluations based on stress tolerance

Developing bioethanol CBP processes not only requires that the host strain can produce cellulases and high ethanol yields and titres, but also that it has tolerance to the harsh conditions found in this fermentation setting (Deparis *et al.*, 2017). Tolerance of strains towards environmental stresses found in the bioethanol production process is thus a major factor to consider when selecting a host strain (Davison *et al.*, 2016). Strains isolated from harsh environments that have evolved to survive stressful conditions, often tend to be more tolerant to numerous stresses, compared to industrial and laboratory isolates that are manipulated to become tolerant using adapted evolution or metabolic engineering techniques. Therefore, we tested if the natural isolates were able to grow in the presence of stressors commonly found in

2G bioethanol production, and how heterologous expression of the cellulases may have changed these tolerances.

3.4.1. Fermentation-associated stressors

A major hurdle in CBP processes is the difference in the optimal temperatures of cellulases and that of the fermentative microorganism(s), corresponding to 45-55°C and 25-35°C, respectively (Branco *et al.*, 2019; Deparis *et al.*, 2017; Shen *et al.*, 2020). Thus, to achieve an efficient and sustainable production process, fermentative microorganisms with increased thermo-tolerance is required. Thermo-tolerant hosts would not only allow for improved simultaneous enzymatic hydrolysis and fermentation of the substrate but would also have a significantly smaller cooling expense and reduced contamination. Therefore, in this study, the thermo-tolerance of wild-type and transformed strains were evaluated by cultivation at 30°C and 40°C, as illustrated in Figure 3.7. All wild-type isolates screened were able to exhibit active growth at both tested temperatures (Figure 3.7A and B), as indicated by the various 10-fold dilutions per isolate. However, transformed natural isolate, YI59_BECC, was noted to be more sensitive to high temperature exposure, as demonstrated by the lack of growth at higher dilutions. This agreed with the lower cellulases secretion capacity of this strain at 37°C (Figure 3.3A, 3.4A, and 3.5A). This suggested that the YI59-based strain might not perform well in fermentation settings with fluctuating temperatures, as the strain already experienced compromised secretion capacity of the heterologous enzymes, increasing the burden on normal cell metabolism.

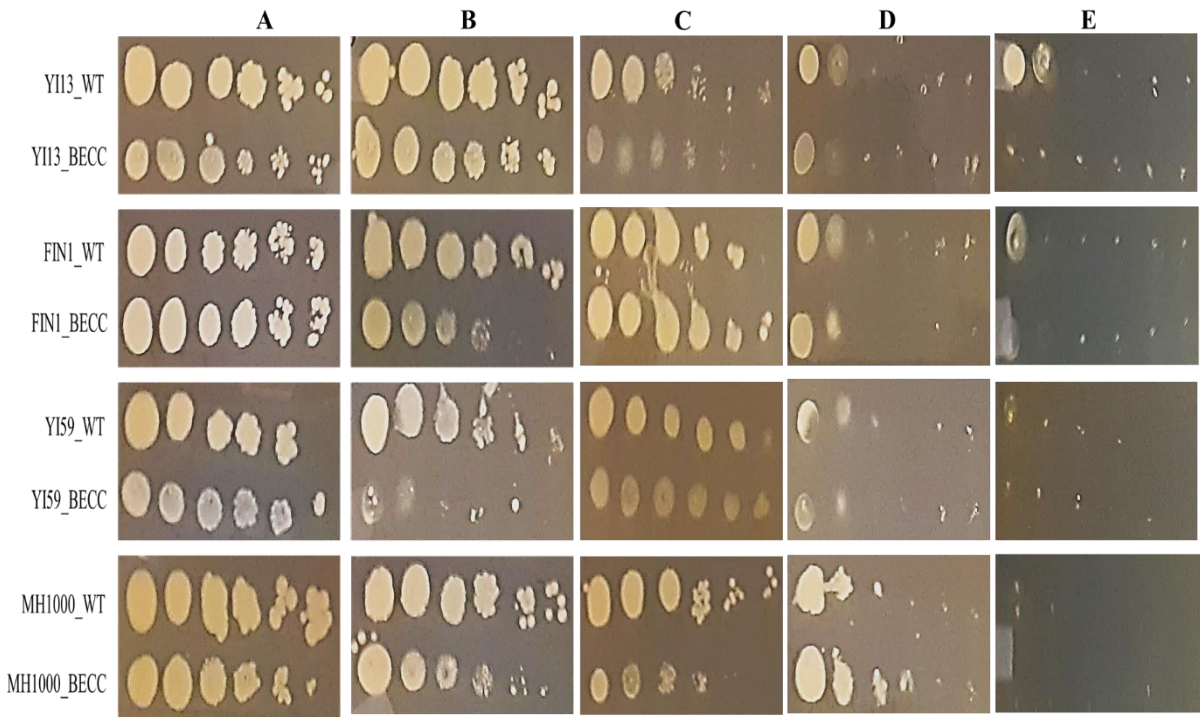

Figure 3.7: Tolerance of isolates against common bioethanol process-related stresses. Isolates were cultivated on YPD media supplemented with an appropriate inhibitor for 48 hours at 30°C, unless otherwise noted. Incubation at (A) 30°C; (B) 40°C; (C) evaluation of tolerance towards 5 g/L acetic acid; (D) evaluation of osmo-tolerance by cultivation on 1.2 M NaCl; and (E) evaluation of strain tolerance to the presence of an 8% (w/v) ethanol. Dilutions are read from left to right, with a starting optical density of OD600nm = 1, followed by 10-fold dilutions thereafter.

Weak acids, such as acetic acid, commonly found in pre-treated lignocellulosic hydrolysates exhibit antimicrobial properties that may negatively affect yeast cell growth and metabolism (Ribeiro *et al.*, 2021). Even though yeast cells also produce acetic acid as part of their normal cellular metabolism, high concentrations of the compound may prove lethal (Chen *et al.*, 2016; Ribeiro *et al.*, 2021). As illustrated in Figure 3.7C, strains were cultivated on YPD solid media supplemented with acetic acid (5 g/L). As expected, YI59_WT and YI59_BECC showed the most robust growth for both wild-type and transformed variants, which agreed with the latter's higher secretory capacity under these conditions (Figures 3.3A, 3.4A, and 3.5A). Furthermore, YI13_BECC, FIN1_BECC, and MH1000_BECC also grew well in the presence of this inhibitor, which may suggest that pre-cultivation of the strain isolates at 30°C prior to inoculating on the YPD solid media containing the inhibitor allowed cells to mitigate the effect of acetic acid. Relating back to the secretory capacity of these strains, these growth patterns may suggest that the burden exerted on the secretion pathway of these strains may have not directly affected their ability to grow in the presence of the inhibitor. However, the secretion

pathway may be suppressed under these conditions, limiting the deleterious effect of acetic acid on the growth of the isolates.

Pre-treatment and/or detoxification of lignocellulosic hydrolysates often results in the introduction of high salt concentrations in 2G bioethanol production processes (Deparis *et al.*, 2017). These high salt concentrations have been shown to alter fermentation rates and ultimately bioethanol yields, as salt exerts an inhibitory effect on yeast growth. Therefore, the viability of wild-type and transformed isolates were evaluated in the presence of 1.2M NaCl, as illustrated in Figure 3.7D. For all isolates screened, moderate to low growth was observed, as illustrated by growth only at lower dilutions for each strain. No growth was observed for isolates at higher dilutions, suggesting that the viability of cells was too low to withstand the salt concentrations. An exception existed for MH1000_BECC, which showed more robust growth than the other isolates, indicating increased tolerance to high salt concentrations.

Possibly the main factor to consider when choosing hosts for CBP processes is the ability to produce and tolerate high ethanol titres to prevent end-product inhibition (Deparis *et al.*, 2017). Ethanol toxicity has been shown to affect the membrane composition of cells, particularly by increasing membrane permeability and/or fluidity, which may influence cell growth and viability (Caspeta *et al.*, 2015). In addition, the reduced water activity may cause a reduction in glycolytic enzyme activity, resulting in suppression of yeast metabolism (Deparis *et al.*, 2017). Having strains with increased tolerance to high ethanol concentrations may thus significantly increase the production capacity of industrial processes. Screening our strains for their tolerance towards high ethanol concentrations (8% w/v) revealed no significant variation in ethanol tolerance (Figure 3.7E). As illustrated, the wild-type variants for YI13 and FIN1 managed to yield some cellular growth at the lower dilution(s); however, low levels of growth were observed. Based on the increased sensitivity displayed by these strains, they may not be robust enough to be used in fermentation settings requiring high ethanol yields and titres. Improvements in ethanol tolerance could be achieved by using adaptive evolution techniques or via metabolic engineering.

3.4.2. Secretion- and cell wall-associated stressors

Secretion of heterologous proteins exerts extensive stress on host cells, which may have the potential to reduce biomass and product yields (Davison *et al.*, 2016; 2019). In *S. cerevisiae*, overexpression of heterologous proteins often results in hyper-glycosylation, which ultimately leads to the misfolding of proteins and the subsequent activation of the UPR pathway.

Furthermore, the UPR pathway is also associated with host cell wall integrity and a connection with tolerance to UPR induction and successful protein secretion was established (Davison *et al.*, 2019). Thus, to evaluate tolerance against ER and cell wall stressors, the chemical compounds Tunicamycin and Congo Red were used to mimic stresses (Figure 3.8).

Tunicamycin is known for blocking *N*-glycosylation of secreted proteins, causing induction of the UPR pathway (Dudgeon *et al.*, 2008), which may ultimately lead to inhibition of cell growth due to cell cycle arrest. This would suggest that strains exhibiting sensitivity towards Tunicamycin might have poor folding and/or post-translational capacity which would therefore correlate with low secretory capacities for the heterologous cellulases as demonstrated by Davison and co-workers (2019). Screening our strains indicated moderate to high sensitivity to the secretion stressor, as illustrated in Figure 3.8A. Wild-type isolates, YI59_WT and MH1000_WT, exhibited more tolerance to Tunicamycin, compared to their transformed variants (i.e., YI59_BECC and MH1000_BECC). This suggested that heterologous protein production in the transformed isolates exerted an increased metabolic burden on the strain isolates. In addition, YI13_WT and YI13_BECC experienced severe sensitivity to the stressor, as little growth was obtained. This was a surprising result, as YI13_BECC was identified to have superior secretory capacity for all the heterologous cellulases assayed for (Figures 3.3A, 3.4A, and 3.5A). Furthermore, the YI13 strain was previously reported to have notable tolerance toward Tunicamycin (Davison *et al.*, 2019). The concentration of Tunicamycin used in this study may have been too high for the strains.

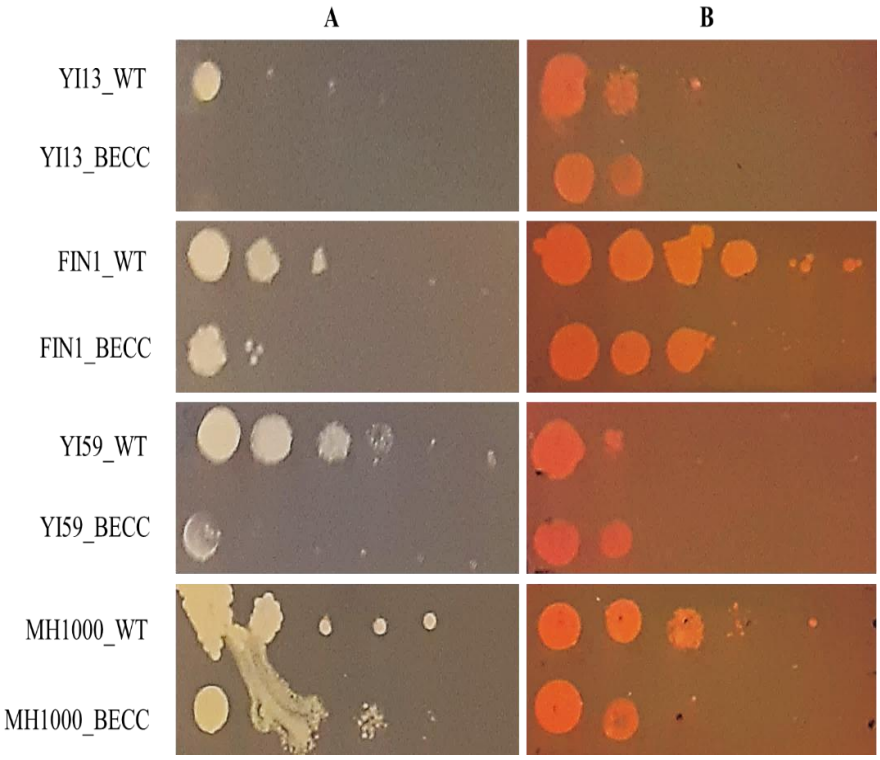

Figure 3.8: Evaluating tolerance towards endoplasmic reticulum (ER) and cell wall stressors. (A) Tunicamycin (1 µg/mL) and (B) Congo Red (600 µg/mL) containing plates were inoculated with the strain isolates and incubated at 30°C for 48 hours. Dilutions are read from left to right, with a starting optical density of OD600nm = 1, followed by 10-fold dilutions thereafter.

Another common stress experienced by yeast cells is alterations to cell wall integrity, often due to high ethanol concentrations (Caspeta *et al.*, 2015; Udom *et al.*, 2019). Exposure to high ethanol concentrations often results in changes in the composition of cell membranes that may result in altered cell growth and viability (Caspeta *et al.*, 2015). To mimic the stress altering cell wall integrity, Congo Red (CR) is often used. As illustrated in Figure 3.8B, moderate to high sensitivity towards CR (600 µg/ml) was noted for both wild-type and transformed strains. This observation agreed with the high ethanol sensitivity obtained (Figure 3.7E). This suggested that despite the diverse strain backgrounds of the isolates screened, their membrane compositions did not allow for tolerance towards high concentrations of ethanol.

3.5. Growth analysis

S. cerevisiae is well known for its use as a recombinant cell factory to produce various proteins of interest; however, heterologous protein production often exerts cellular and metabolic stresses on the yeast cells (Davison *et al.*, 2016; Deparis *et al.*, 2017; Ilmén *et al.*, 2011). These changes in the physiology of the yeast can result in obtaining low protein production levels, due to impaired growth and metabolism. Due to the variation in protein production levels

achieved in this study, growth profiles of the strains were evaluated to determine the effects of heterologous cellulase production on growth.

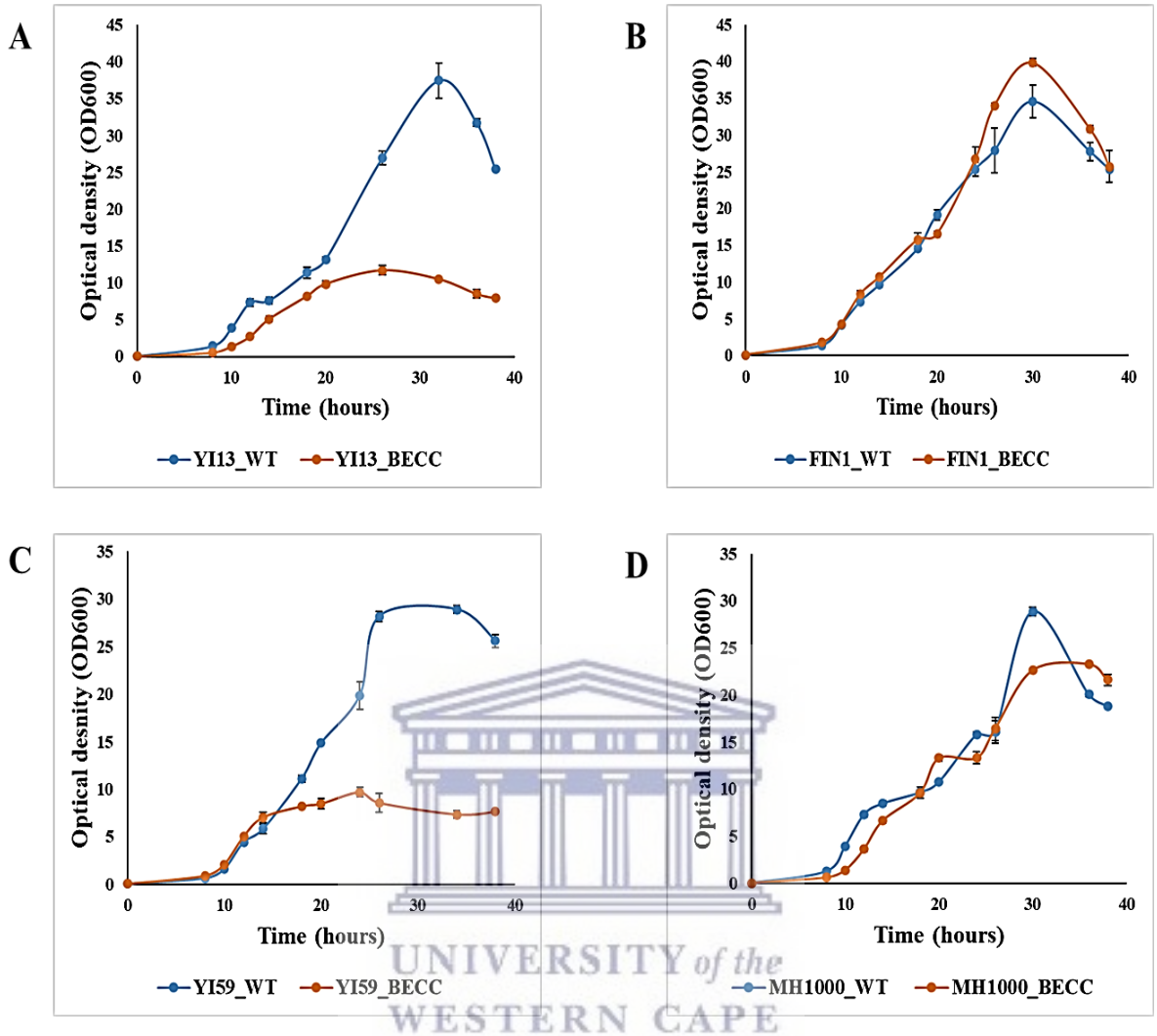

Figure 3.9: Growth curves of (A) YI13, (B) FIN1, (C) YI59, and (D) MH1000, wild-type and transformed strains on YPD at 30°C. Data points represent the mean of three biological repeats per respective strain isolate, and error bars indicate mean ± standard deviation.

As illustrated in Figure 3.9, growth profiles for YI13, FIN1, YI59, and MH1000 wild-type and transformed strains were evaluated in YPD liquid media at 30°C over the course of 39 hours. No significant variation could be observed between the wild-type and transformed isolates of FIN1 (Figure 3.9B) and MH1000 (Figure 3.9D). However, it was noted that the growth of YI13_BECC (Figure 3.9A) and YI59_BECC (Figure 3.9C) were significantly impaired compared to their untransformed counterparts. This suggested severe metabolic burden because of constitutive expression of the cellulases. As the YI13_BECC and YI59_BECC strains generally produced high enzyme activity levels (Figures 3.3-3.5), we assume they are exhibiting higher protein production levels at the expense of biomass production.

3.6. Fermentation of crystalline cellulose

Direct conversion of a crystalline cellulose substrate (Avicel) to ethanol in a CBP configuration without the addition of exogenous cellulases was tested using our strains. Ethanol production was tested, by inoculating pre-cultured strains on Avicel (20 g/L) in oxygen-deficient conditions for 120 hours. Samples were taken for HPLC analysis (Figure 3.10; Table 3.1).

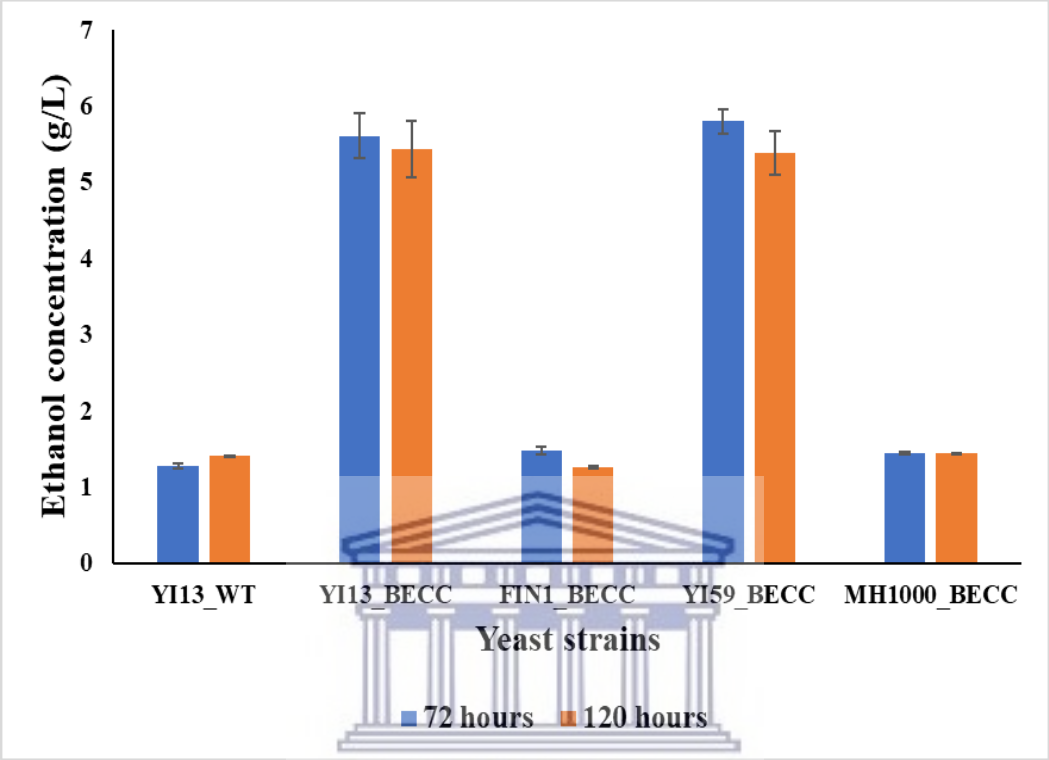

Figure 3.10: Ethanol titres attained by strains cultivated on 20 g/L Avicel for 72 and 120 hours. Data bars represent the mean of three biological repeats, and error bars represent mean ± standard deviation.

Figure 3.10 shows that ethanol concentrations in the range of ~1-5.5 g/L was obtained. YI13_BECC and YI59_BECC were the best ethanol producers in this CBP conversion of cellulose to ethanol, generating 35-40% of the theoretical maximum ethanol yield. This agreed with enzyme activity evaluations illustrating that these two strains were the superior secretors for cellulases, suggesting the secreted enzymes were active and functional to have achieved efficient Avicel hydrolysis. Interestingly, the MH1000_BECC strain did not achieve ethanol levels that were significantly higher than that of the control strain as would have been expected based on its significant Avicel hydrolysis (Figure 3.6). This might suggest that additional stress factors of the CBP set up prevented significant ethanol production by this strain. It should be noted that a large volume of pre-culture was used to inoculate these fermentations to allow for carry over of secreted cellulases. This would have led to some ethanol from the pre-culture

being carried over to the fermentation cultures, explaining the ~1 g/L amounts of ethanol observed for the negative control strain, YI13_WT.

Glycerol is often produced in response to high osmolarity as a result of increasing sugar concentrations, or in response to increased ethanol concentrations (Pérez-Torrado *et al.,* 2016; Shokoohi *et al.,* 2016). Production of glycerol helps to protect cells against high sodium salt stress by dispersing the driving force for sodium ion uptake across the plasma membrane to the interior of cells. In addition, glycerol also aids in protecting cells against dehydration caused by high osmolarity and ethanol toxicity, by restoring turgor pressure and cell functioning. It can therefore be said that glycerol plays a critical role in osmoregulation and protection of cellular enzymatic activities under salt and ethanol stress. As shown in Table 3.1, glycerol levels of less than 1 g/L were produced by YI13_BECC, YI59_BECC, and MH1000_BECC. This may have occurred as a result of increasing sugar or ethanol concentrations during the fermentation. No detectable residual glucose was present at 120 hours in any of the fermentations. This suggested that all the liberated glucose was utilised by the fermentative isolates. Residual cellobiose levels obtained were in the range of 0.82-1.47 g/L, suggesting EG and CBH were functional. However, the notable levels of cellobiose present at 120 hours may suggest that the BGL activity was not high enough, hence, EG and CBH activity may also have been inhibited to some extent. It is also possible that the Michaelis constant (K_M) for the BGL in these conditions may be higher than the cellobiose levels observed, leading to liberated cellobiose and oligosaccharides that were not converted to monomeric glucose. Lastly, observed acetic acid levels in the range of 0.67-1.29 g/L may suggest production of the weak acid to neutralise the extracellular pH (Deparis *et al.,* 2017).

Table 3.1: Metabolites produced by strains cultivated on 20 g/L Avicel for 120 hours. Values are given as g/L, together with standard deviation per triplicate biological repeat for each strain.

Strains	Cellobiose	Glycerol	Acetic acid
YI13_WT	0,852 ± 0,003	0,621 ± 0,016	0,667 ± 0,048
YI13_BECC	1,302 ± 0,060	0,892 ± 0,057	1,013 ± 0,063
FIN1_BECC	0,818 ± 0,002	0,598 ± 0,004	0,701 ± 0,044
YI59_BECC	1,371 ± 0,060	0,730 ± 0,079	1,291 ± 0,032
MH1000_BECC	1,470 ± 0,027	0,787 ± 0,005	0,725 ± 0,015

Based on the fermentation profiles obtained for the natural isolates and the reference strain, it can clearly be seen that YI13_BECC and YI59_BECC were better equipped to secrete the heterologous cellulases, and still produce relatively high ethanol titres. This suggested the

potential for use of these strains in a CBP setting. To mitigate exposure and hence sensitivity towards increasing sugar concentrations, these strains were able to produce glycerol and acetic acid at higher levels than FIN1_BECC and MH1000_BECC, making them more robust fermentation hosts.

3.7. Discussion

Overexpression of heterologous proteins may result in a severe metabolic burden on cellular hosts, as energy and resources are redirected from normal metabolism to heterologous gene expression and enzyme production (Brandt *et al.,* 2021). The extent of the metabolic burden in recombinant cellulase producing strains is dependent on various factors, such as gene copy number, source of the gene, the expression strategy (i.e., secretion, cell-attachment, mini-cellulosomes, and/or combinations), oxygen availability, and the strain background. In addition, changes in the physical environment (temperature, pH, nutrients, solutes, and inhibitors) also contribute significantly to the viability of cells and the resulting heterologous protein production process (Brandt *et al.,* 2021; Shen *et al.,* 2020). We therefore investigated heterologous cellulase production in various strain backgrounds, previously shown to be robust to various CBP related stress factors, to determine how process relevant changes in the environment, which impact these strains differently, affected their ability to secrete recombinant enzymes.

Like all (micro)organisms, yeast cells have a temperature range (minimum, optimal, and maximum) in which it can function to ensure physiological activity in cells (Shen *et al.,* 2020). Under nutrient-rich conditions, yeast cells grow optimally in the temperature range 25-30°C. An increase in temperature above 36°C might induce a stress response, referred to as heat shock. To mitigate the effect exerted by high temperature exposure, cells might induce expression of heat shock proteins (HSPs) and/or accumulation of trehalose, which function by preventing protein aggregate formation or stabilising normal cellular proteins and membranes (Shen *et al.,* 2020; Verghese *et al.,* 2012). As discussed in subsections 3.2.1-3.2.3, YI13_BECC was noted to have superior secretory capacity for heterologous cellulases when cultivated under high temperature conditions (37°C), which may allude to increased tolerance to high temperature by this strain, potentially acquired from the vineyard environment it was isolated from. This increased thermo-tolerance was further confirmed by stress plate assays (Figure 3.8B), which showed that YI13_BECC exhibited robust growth even when cultivated at 40°C. Furthermore, the high Avicel conversion efficiency obtained by this strain demonstrated

production of secreted heterologous cellulases that were active and functional to allow synergistic action on the Avicel substrate (Figure 3.6B). The growth rate of YI13_BECC may have also contributed to the reduced temperature sensitivity, as slow growing cells (shown in Figure 3.9A) are much less affected by heat stress (Gibney *et al.*, 2013). However, despite the increased tolerance to high temperatures, YI13_BECC displayed increased sensitivity to the additional process condition stresses tested for (Figure 3.7C-E).

During lignocellulosic bioprocessing, various stresses exist that have adverse effects on yeast cell growth and viability (Brandt *et al.*, 2021). An example of such stress is acetic acid, which is a major inhibitory compound found in lignocellulosic hydrolysates, and which is also produced during normal yeast metabolism (Ribeiro *et al.*, 2021). Acetic acid in its undissociated form can diffuse across the plasma membrane of yeast cells to lower the intracellular pH within cells. This leads to accumulation of acetate counter-ions, increased turgor pressure, oxidative stress and inhibition in cell metabolism. To pump the excess protons from the cell to regulate the redox balance would thus be done at the expense of ATP hydrolysis which ultimately arrests cell growth and reduces performance of the yeast cells within the fermentation setting (Chen *et al.*, 2016; Ribeiro *et al.*, 2021). For this reason, secretion titres of the heterologous cellulases in natural isolates and reference strains were investigated in acetic acid-stressed cells to evaluate how the secretory pathway of the yeast was affected. As discussed in subsections 3.2.1-3.2.3, YI59_BECC displayed superior secretory capacity for all heterologous cellulases assayed for under acetic acid stress. Based on available transcriptomic, proteomic, and chemogenomic data, the underlying mechanism for increased tolerance to acetic acid stress in this strain isolate could potentially be explained by changes in the physical properties and molecular composition of the plasma membrane and cell wall to allow for a decrease in the permeability of the cell envelope (Ribeiro *et al.*, 2021). This would suggest that YI59_BECC counteracted the diffusion rate of the weak acid from the culture medium into the cell interior. As a result, normal cellular metabolism could continue, and the secretion pathway was not negatively affected. Increased robustness by YI59_BECC against acetic acid stress was further confirmed by cultivation on 5 g/L acetic acid (Figure 3.7C) and the superior Avicel conversion efficiency obtained under the same stress (Figure 3.6C).

To realise the implementation of CBP in bioethanol production, microorganism(s) capable of producing active cellulolytic enzymes and utilising both hexose and pentose sugar monomers, whilst tolerating toxic biomass hydrolysates and fermentation process conditions, is required (Olguin-Maciel *et al.*, 2020; Valenzuela-Ortega and French, 2019). From the results obtained,

it was noted that natural isolates of *S. cerevisiae* offer great potential for use as hosts for heterologous cellulase production under various process conditions. However, we also noted that the tolerances of transformed natural and reference strains to inhibitory compounds found in pre-treated lignocellulosic hydrolysates and fermentation process settings, as previously reported by Davison and co-workers (2016), were significantly affected. In our study, wild-type and transformed strains exhibited low tolerance to high sodium salt and ethanol concentrations, while active to moderate growth was previously observed for strains expressing individual cellulases in the same background strains (Davison *et al.*, 2016). The decrease in strain tolerance likely resulted from the constitutive expression of multiple heterologous cellulases in the transformed isolates, which exerted a severe metabolic burden on the yeast cells resulting in impaired cell growth and viability (Caspeta *et al.*, 2015; Deparis *et al.*, 2017). Similarly, our strains displayed sensitivity to Tunicamycin and Congo Red, compared to more robust growth in the reported study (Davison *et al.*, 2016). This was surprising, as YI13_BECC and YI59_BECC was noted to exhibit superior secretory capacities under the process conditions assayed for in this study, suggesting proper folding and post-translational modifications of heterologous cellulases were achieved in these strain backgrounds. Therefore, it could be proposed that low tolerance to Tunicamycin and Congo Red were displayed by these strains, due to the increased concentrations of stressors used in this study. In addition, the slow growth of cells (Figure 3.9A and C) may have caused a delayed growth pattern as longer adaptation periods (lag phase) may have been required in these conditions.

Comparison of heterologous cellulase production under optimal and stressed conditions demonstrated the diversity in strain backgrounds, as the strains exhibited varying tolerances and secretory capacities in various cultivation conditions, emphasising the differences between the strains. Fermentation profiles of transformed natural and industrial strains demonstrated the potential use of YI13_BECC and YI59_BECC as CBP hosts for bioethanol production, with ethanol concentrations of 4-4.5 g/L obtained when cultivated on 20 g/L Avicel for 120 hours. Therefore, we can surmise that integration of the core cellulase enzyme complex into these strain backgrounds, with the use of CRISPR/Cas9 tools, allowed for the construction of superior recombinant cellulolytic hosts. Compared to recombinant strains constructed via conventional transformation strategies, the CRISPR/Cas9-constructs demonstrated sufficient capacity to heterologously produce cellulases to liberate glucose for fermentation to ethanol, while tolerating the harsh environmental conditions posed in the fermentation setting.

CHAPTER 4: SUMMARY AND CONCLUSION

The production of cellulosic bioethanol has gained much attention over the past two decades, due to lignocellulosic biomass being inexpensive and abundantly available in large quantities. However, due to the recalcitrant nature of lignocellulosic biomass, microorganisms with both cellulolytic activity and high ethanol-productivity are required for an efficient bioethanol production process. The aim of this study was to endow natural *S. cerevisiae* isolates with a basic cellulolytic system, by introducing genetic modifications into the yeast genome via CRISPR/Cas9 tools. In addition, strain tolerance to inhibitors commonly found in fermentation processes and lignocellulosic hydrolysates were also investigated, to identify host strains with increased robustness. It was hoped that by improving heterologous cellulase production and increasing strain tolerance to varying inhibitory conditions, cellulose CBP strains could be obtained for potential application in 2G bioethanol production.

We successfully transformed natural and industrial *S. cerevisiae* isolates with cellulase-encoding genes, using CRISPR/Cas9 tools. Initially, low BGL (*S.f.BGLI*) activity was observed in our transformants despite the gene being under control of different strong constitutive promoter sequences (*PGK1*$_P$ or *ENO1*$_P$). Newly obtained transformants yielded significantly improved BGL (*A.a.BGLI*) activity when expressed under the *SED1*-promoter and *DIT1*-terminator regulatory sequences. Subsequently, we evaluated the enzyme activity of a core cellulase complex (CBHI, CBHII, EG & BGL) in various strain backgrounds, and we showed that YI13_BECC exhibited superior secretory capacity when cultivated under nutrient-rich conditions as well as at elevated temperatures. However, YI59_BECC exhibited superior secretory capacity for the cellulases in the presence of acetic acid, which illustrated the genetic diversity among our strain backgrounds. Avicel hydrolysis assays showed superior conversion efficiencies by YI13_BECC and YI59_BECC in the same cultivation conditions, which agreed with the results obtained from enzyme activity assays. Growth analyses demonstrated reduced growth patterns for the superior cellulase secretors, which suggested that superior heterologous cellulase production was attained at the expense of biomass production. Comparing untransformed and transformed natural and industrial *S. cerevisiae* strains in terms of their tolerance to industrial and secretion stresses, showed that natural isolates exhibited higher innate tolerances with increased robustness, especially in YI13_BECC and YI59_BECC.

We subsequently attempted a rudimentary CBP fermentation with our heterologous cellulase producers on crystalline cellulose (Avicel). Encouragingly, ethanol concentrations of up to 4.5

g/L were obtained by YI13_BECC and YI59_BECC, which correlated to a 35-40% of the theoretical maximum ethanol yield. We concluded that the transformed strain isolates offer significant potential for use in 2G bioethanol production.

4.1. Future perspectives

Due to the marker-less nature of CRISPR/Cas9 methods, the promising strains constructed in this study could further be optimised through addition and/or knock-outs of genes implicated in more robust, stress tolerant phenotypes. Constructing CBP hosts with increased tolerance to high temperatures and other stresses to allow optimal functioning of heterologous cellulases can also be conducted with genome shuffling techniques (Mitsui *et al.*, 2019). Additionally, activation and/or repression of gene expression of cellulase-encoding genes can be regulated using CRISPR/dCas9 systems (Ding *et al.*, 2020). Furthermore, classical breeding of these superior secretors, to obtain offspring that exhibit superior heat and acetic acid tolerance, as well as optimal cellulase ratios is another interesting option to pursue in future. By mating haploid strain derivatives exhibiting superior stress tolerance and secretion capabilities, diploid derivatives can be obtained that exhibit enhanced CBP characteristics (Kroukamp *et al.*, 2017). Although stress responses have been shown to be strain dependant, data obtained from omics approaches and genetic engineering techniques could aid in further optimisation of the biological capabilities of natural *S. cerevisiae* strains to allow for the construction of CBP hosts for 2G bioethanol production.

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APPENDIX A: SUPPLEMENTARY FIGURES

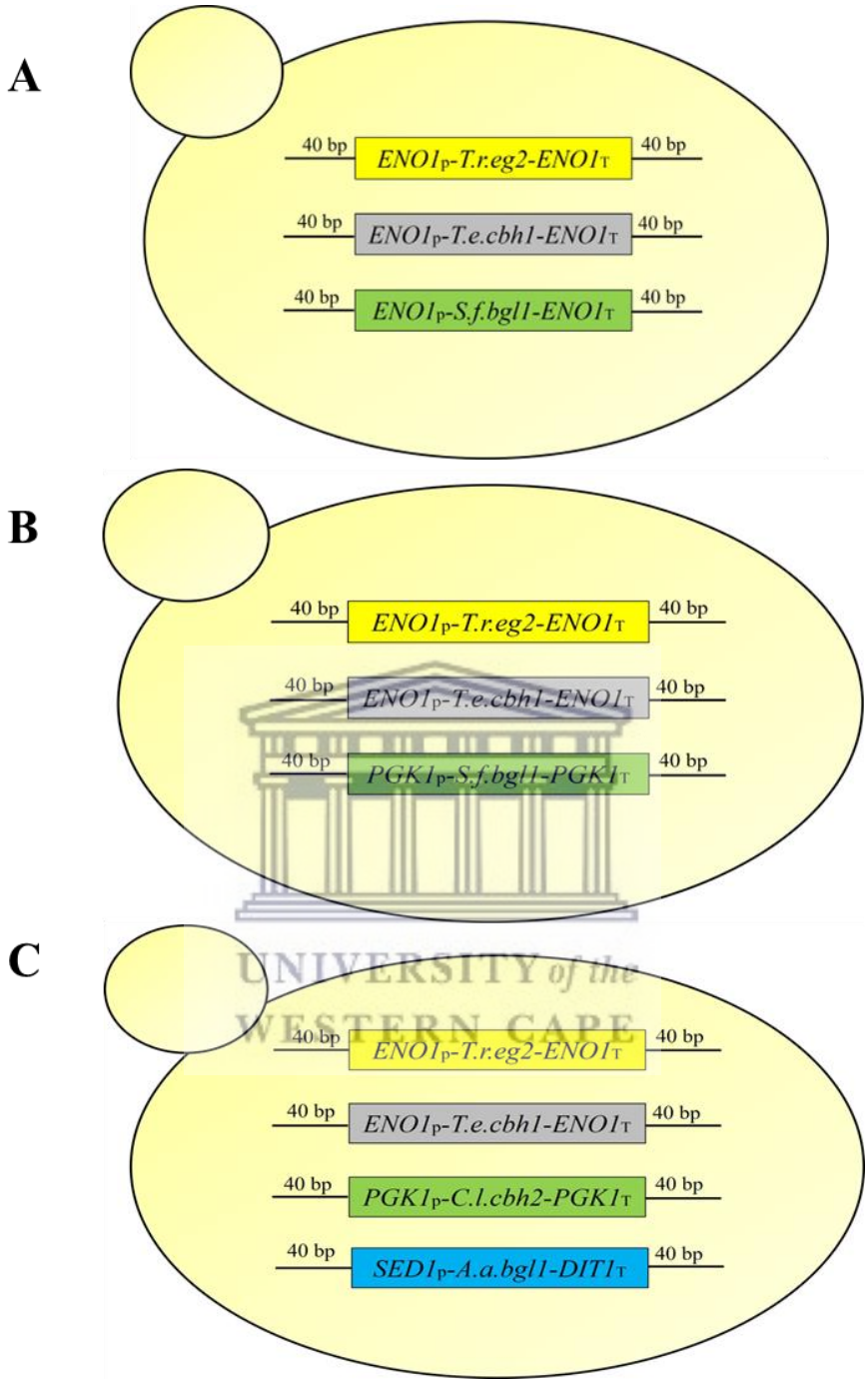

Figure S1: Graphical representation of natural and industrial *Saccharomyces cerevisiae* strains constructed in this study. With successive rounds of transformation, cellulase-encoding genes were integrated into the genome of the background strains. Different combinations for each strain isolate were constructed (i.e., A- ECBE, B- ECBP, and C-BECC), with distinction between them based on the regulatory promoter sequence used for expression of the beta-glucosidase (BGL)-encoding gene. Each gene cassette was also equipped with a 40bp overhang that shared homology with the host genomic sequence, where pCas9 generated the double-stranded break (DSB).

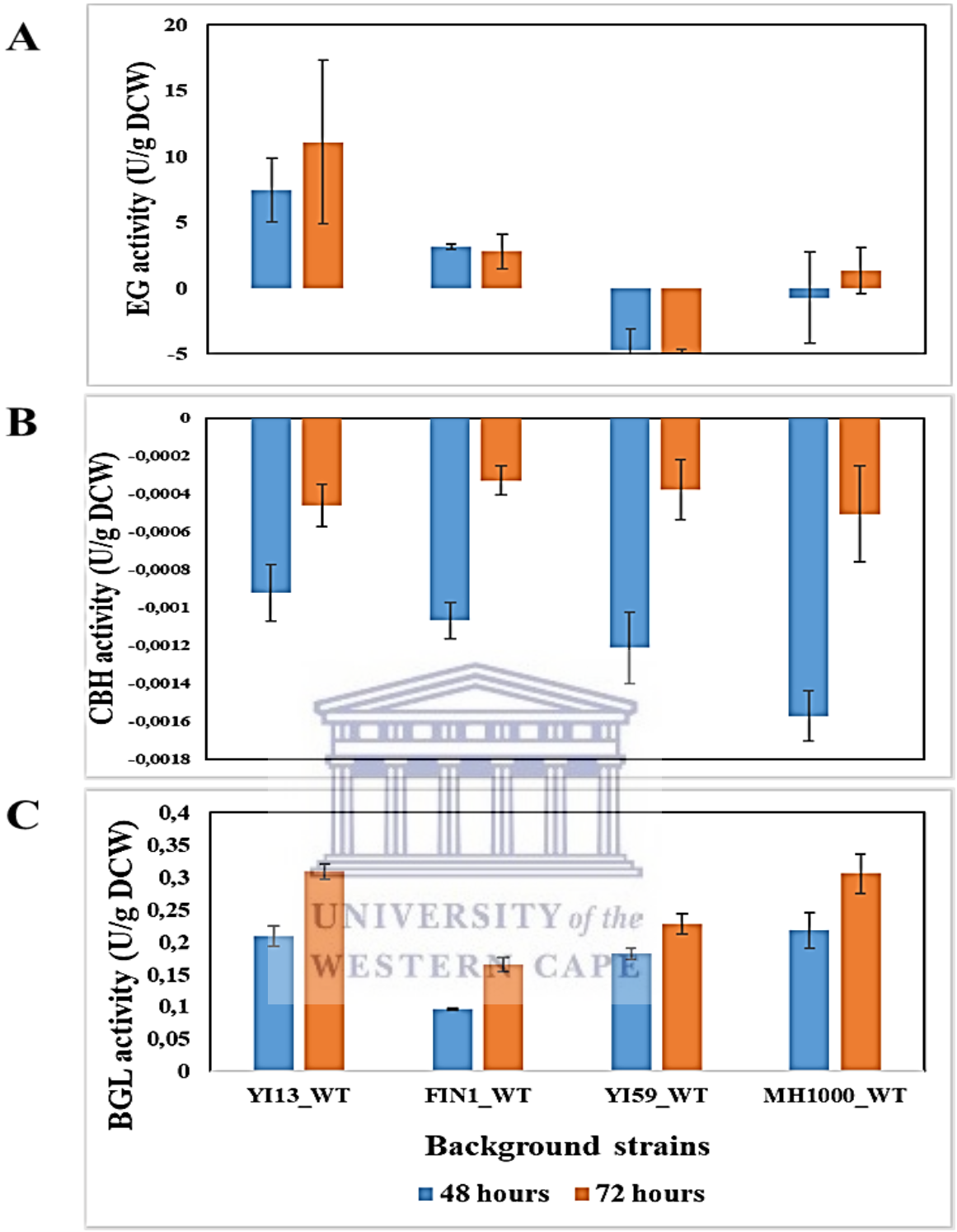

Figure S2: Enzyme activity evaluations of background strain in various background conditions. Yeast strains were cultivated in 10 ml YP media supplemented with 2% (v/v) glucose for 72 hours at 30°C, prior to evaluation of (A) endoglucanase (EG), (B) cellobiohydrolase (CBH), and (C) beta-glucosidase (BGL), activity levels. Volumetric values (U/L) obtained were standardised with dry cell weight (DCW) of each respective isolate in g/L. Data bars represent the average of three biological repeats per strain, and error bars represent the mean ± standard deviation.

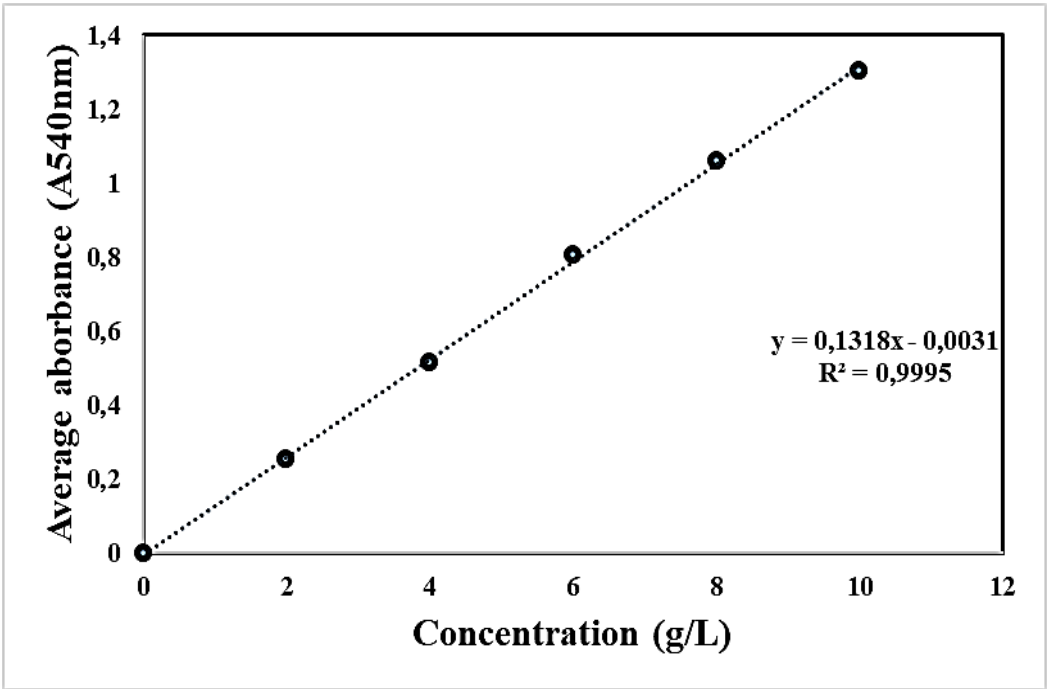

Figure S3: Dinitrosalicylic acid (DNS) standard curve used to determine unknown concentrations of reducing sugars liberated during endoglucanase activity measurements.

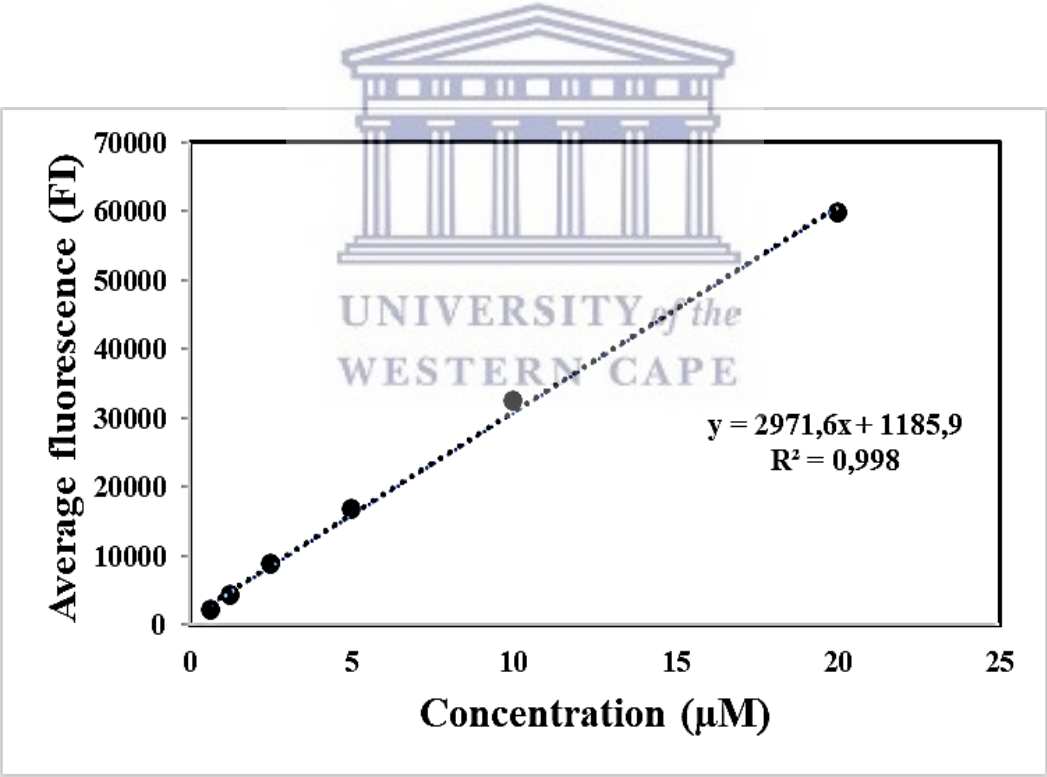

Figure S4: 4-Methylumbelliferyl standard curve used to determine unknown concentrations of methylumbelliferyl released by action of cellobiohydrolase I.

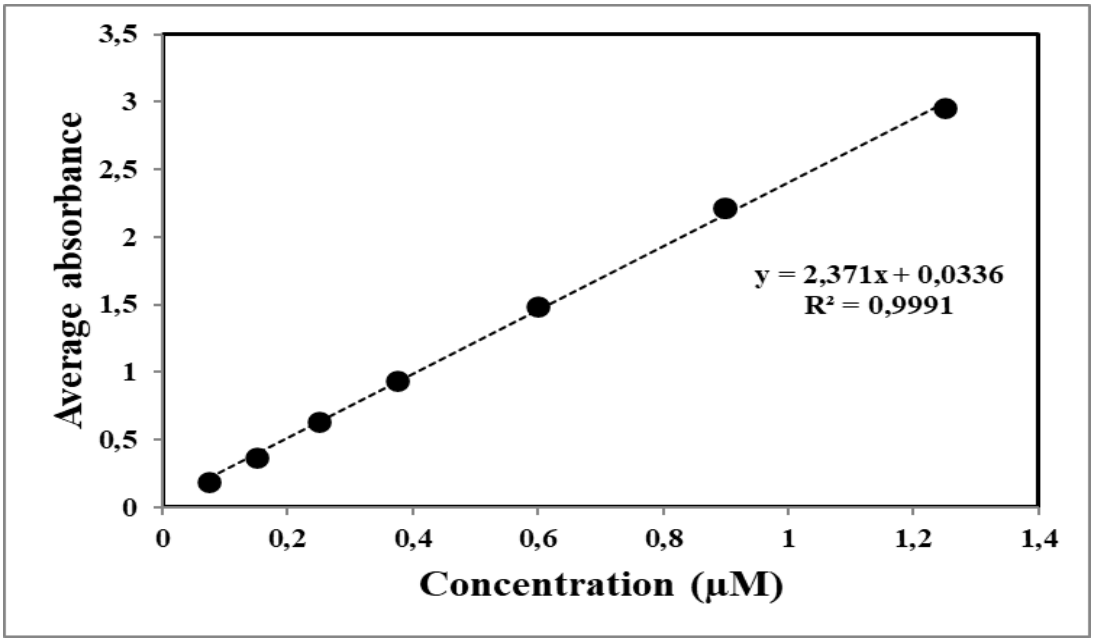

Figure S5: 4-Nitrophenyl standard curve used to determine unknown concentrations of 4-NP released by action of β-glucosidase I.

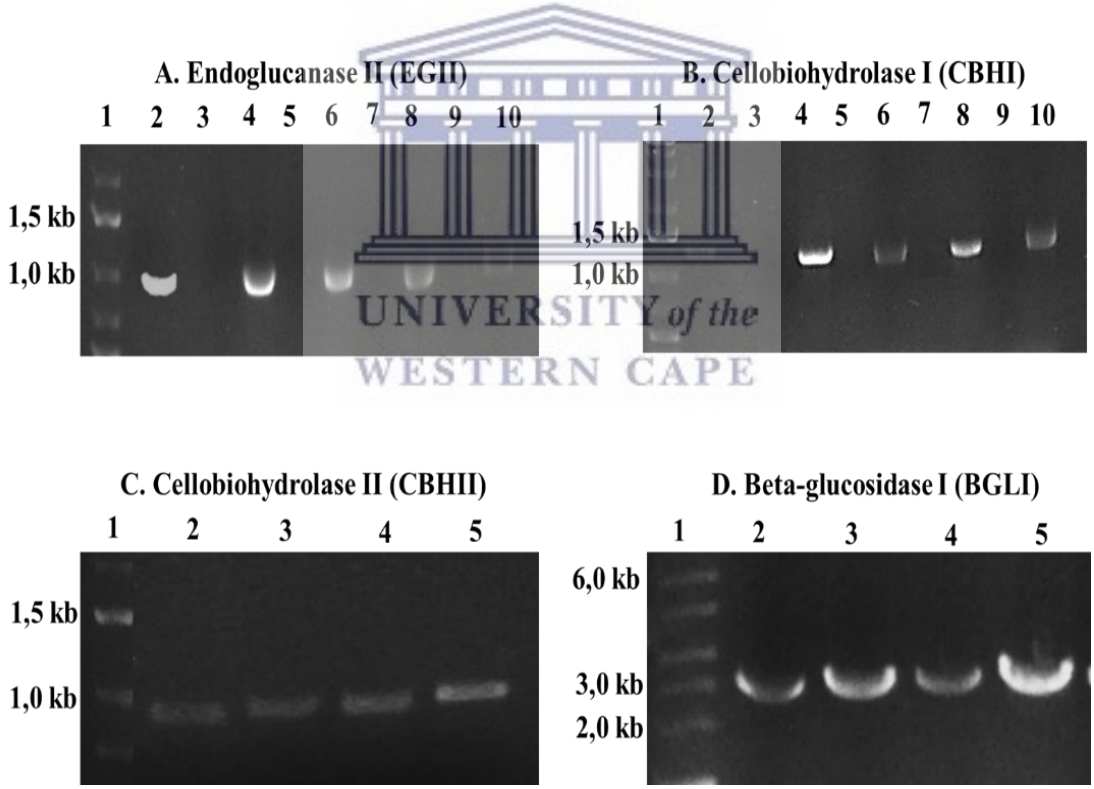

Figure S6: Gene integration confirmations. (A) Endoglucanase (EGII) and (B) Cellobiohydrolase (CBHI) was confirmed for both negative and positive transformants. Lane 1: Marker (1 kb Safe Stains, NEB), Lane 2: (a) Y294+EGII/(b)Y294+CBHI, Lane 3: YI13_WT, Lane 4: YI13_EGII/CBHI, Lane 4: FIN1_WT, Lane 5: FIN1_EGII/CBHI, Lane 6: YI59_WT, Lane 7: YI59_EGII/CBHI, Lane 8: MH1000_WT, Lane 9: MH1000_EGII/CBHI. (C) Cellobiohydrolase (CBHII) and (D) Beta-glucosidase (BGL) was also confirmed for both negative and positive transformants. Lane 1: Marker (1 kb Safe Stains, NEB), Lane 2: YI13_CBHII/BGLI, Lane 3: FIN1_CBHI1/BGLI, Lane 4: YI59_CBHII/BGLI, Lane 5: MH1000_CBHII/BGLI.