Engineering robust yeast strains for the conversion of xylose derived from lignocellulosic biomass to xylitol

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UNIVERSITY *of the*
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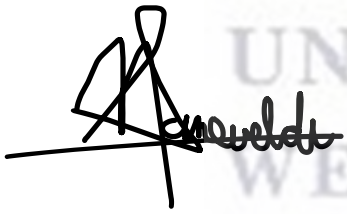

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TABLE OF CONTENTS

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LIST OF ABBREVIATIONS

1G – first-generation

2G – second-generation

AZCL – Azurine cross-linked

BDO – butandiol

BE – bioeconomy

bp – base pair

C5 – five-carbon

C6 – six-carbon

CAGR – compound annual growth rate

Cas9 – CRISPR-associated protein 9

CBE – circular bioeconomy

CBP – consolidated bioprocessing

CE – circular economy

CO_2 – carbon dioxide

CRISPR – clustered regularly interspaced short palindromic repeats

DCW – dry cell weight

DNA – deoxyribonucleic acid

DNS – dinitrosalicylate

DOE – Department of Energy

DP – degree of polymerization

DSB – double-stranded break

DTT – dithiothreitol

EJ – exajoules

EU – European Union

GFN – global footprint network

GH11 – glycosyl hydrolase family 11

GH3 – glycosyl hydrolase family 3

GH43 – glycosyl hydrolase family 43

GHG – greenhouse gas

GI – glycaemic index

GOI – gene of interest

GRAS – generally regarded as safe

gRNA – guide ribonucleic acid

HG – high gravity

LB – Luria-Bertani

LCA – life cycle assessment

LCB – lignocellulosic biomass

LCC – life cycle costing

LCI – life cycle impact

LCSA – life cycle sustainability assessment

LTR – long terminal repeat

LUC – land-use change

MCF – microbial cell factory

Mt – megatonnes

N_2O – nitrous oxide

NAD^+ – nicotinamide adenine dinucleotide

NADH – NAD and hydrogen

NADPH – nicotinamide adenine dinucleotide phosphate

PCI – phenol: chloroform: isoamyl alcohol

PCR – polymerase chain reaction

pNP-X - *p*-nitrophenyl-β-D-xylopyranoside

PPP – pentose phosphate pathway

R&D – research and development

SCFA – short-chain fatty acid

SHF – separate hydrolysis and fermentation

SLCA – social life cycle assessment

SSF – simultaneous saccharification and fermentation

TPE – total protein extract

US – United States

XDH – xylitol dehydrogenase

XI – xylose isomerase

XLN43_SED1 – xylosidase anchored by SED1 anchoring protein

XOS – xylo-oligosaccharides

XR – xylose reductase

XYN1 – endo-xylanase 1

XYN2 – endo-xylanase 2

YP – yeast extract, peptone

YPD – yeast extract, peptone, D-glucose

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

To achieve a sustainable and economically viable 2G biofuels industry, biorefineries must co-produce high-value, low-volume bioproducts alongside high-volume, low-value cellulosic ethanol. This can be realised with the co-production of the low-calorie sugar substitute, xylitol which has a well-established market, as well as other chemicals. The construction of a xylitol-producing *S. cerevisiae* strain represents an economically feasible and environmentally friendly approach to xylitol production. Moreover, the exploitation of natural *S. cerevisiae* strain isolates as bioengineering hosts has the potential to be superior starting points due to their robustness towards process conditions. In this study, the xylitol-producing activities conferred to three natural isolate host strains via conventional and CRISPR-Cas9-mediated δ-integration of three genes encoding a β-xylosidase, β-xylanase and xylose reductase (XR), was evaluated. The effect of over-expressed heterologous protein production on strain robustness and metabolism was also assayed. Our results revealed that the overexpressed XR failed to improve on the xylose reduction ability conferred to our strains, likely by their native *GRE3* gene. The exploitation of natural host isolates proved advantageous, given the high heterologous xylanase and xylosidase activities recorded, far-exceeding previously reported studies, which enabled the substrates xylan and xylo-oligosaccharides (XOS) to be used for xylitol production, instead of costly pure xylose. Despite the high levels of heterologous protein production, our engineered natural strains displayed tolerance to acetic acid concentrations higher than 3 g/L but lower than 5 g/L while FIN1-X3 and YI13-X3 displayed tolerance to temperatures as high as 40 °C. Growth analyses revealed that only YI59-X3 displayed somewhat impaired growth, however, no single strain outperformed the other across the recorded assays of this study. The results of this study led us to conclude that the xylose reduction ability of our strains must be enhanced through alternate genetic engineering strategies. Furthermore, the engineering strategies employed for heterologous xylanase and xylosidase activity as well as the use of natural strains as bioengineering hosts, offer considerable potential for use in 2G biorefineries.

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Fossil fuel reserves have enhanced our quality of life since its discovery as it has met most of our expanding energy and material demands in various sectors of society (Mohan *et al.,* 2019). Sadly, the use of this unsustainable energy source has created significant environmental damage (Vohra *et al.,* 2014). On the 29th of July 2021, the Global Footprint Network (GFN) announced that we had depleted all the resources that our planet could regenerate that year, indicating that we require the regenerative capacity of 1.7 Earths to sustain our consumption (Ho, 2021). Mohan *et al.* (2019) described our current linear economy as "not only unsustainable but also unstable". In brief, the linear economy is based on the *"take-use-dispose"* model which has proved to be ineffective and short-lived (Mazur-Wierzbicka, 2021). This economic model prioritises the production of goods from limited natural resources without considering waste generation which has resulted in waste becoming a worldwide problem. Management of this excessive waste accumulation is costly and has instigated and intensified climate change and global warming (Osorio *et al.,* 2021; Purwanto and Prasetio, 2021). Africa's current linear economy utilises fossil fuels, namely coal, gas, and oil, for energy generation which creates waste materials (excess and used oil, carbon emissions, and fly ash) that are dumped and adversely affect the environment (Mutezo and Mulopo, 2021).

The consequences of the linear economy have forced governments to draft new national policies where the environment is protected, and sustainable development is prioritized (Bloesch *et al.,* 2015). One of the fundamental principles of a sustainable economy is the concept of a circular economy (CE) (Mohan *et al.,* 2019). CE proposes to have materials flow in a circular fashion based on *"resource-product-recycled resources"* (Figure 1.1) (Gil, 2021). Also referred to as a closed-loop economy, it aims to prevent the single-use of materials by (i) closing loops (recycling and remanufacturing), (ii) slowing loops (extending the lifespan of products and goods), and (iii) narrowing loops (utilising natural resources more efficiently within the linear approach) (McCarthy *et al.,* 2018). Globally, the CE framework is projected

to be worth $ 4.5 trillion by 2030 and has the potential to increase (i) consumer savings, (ii) innovation and jobs, (iii) the surety of raw material sources, and (iv) reduce environmental damage (*Quincy Recycle*, 2020).

Figure 1.1. Schematic representation of the flow of materials according to the CE concept: *"resource-product-recycled resources"*. Adapted from (Gil, 2021).

Due to (i) the rise in crude oil prices, (ii) depleting fossil fuel reserves, (iii) concerns over energy security, and (iv) the necessity of climate change mitigation, biofuels have become notable contributors to the global energy supply, thereby supporting the transition towards a CE (Den Haan, 2018; Jeswani *et al.,* 2020; Ranjbari *et al.,* 2022). Biofuels are defined as any replacement fuels originating from biomaterials (Hirani *et al.,* 2018). Bioethanol production has been dominated by the United States (US) and Brazil with production amounting to 1 347 and 883 petajoules in 2020, respectively (Sönnichsen, 2021). Together these two countries account for approximately 87 % of the global biofuel yield (Bajpai, 2021). Consequently, the biofuel feedstocks used by these two countries, namely, corn grain (US) and sugarcane (Brazil), dominate the energy crops for current bioethanol production (Jeswani *et al*., 2020; Ramos *et al*., 2022).

The diverse raw materials used in the production of bioethanol are conveniently organized

into three main categories namely, sugar, starch, and cellulosic materials (Saini *et al.*, 2015). First-generation (1G) biofuels are manufactured from sucrose- and starch-rich food crops. Although sugars can be converted directly to ethanol, starch first requires enzymatic hydrolysis to fermentable sugars (Bajpai, 2021). The use of these food-grade feedstocks comes with many consequences, some of which include: (i) competition with food for arable land summarised by the term *"food vs. fuel"*, (ii) competition for accessible clean water, (iii) costly production expenses, (iv) dependence on unsustainable fertilizers that may restrict greenhouse gas (GHG) mitigations and (v) the encouragement of deforestation for arable land (Kargbo *et al.,* 2021; Mohr and Raman, 2013). During the food crises of 2007-2008, Mitchell (2008) deduced that the rapid surge in globally traded food prices since 2002, was resultant of the production of biofuels from foodgrains and oilseeds in the US and the European Union (EU). Together, these shortcomings render 1G biofuels unsustainable and have led to the establishment of a second generation (2G) of feedstock that is renewable (Ning *et al.,* 2021).

Second-generation biofuels are produced from (i) agricultural and forestry residues or co-products, (ii) cellulosic energy crops, and (iii) woody biomass i.e., non-food materials (Bajpai, 2021; Hirani *et al.,* 2018; Mohr and Raman, 2013). Utilising the inevitable by-products of the agricultural industry as biofuel sources is advantageous since additional fertiliser, land, and water are not required to produce these feedstocks (Dahman *et al.,* 2019). Additionally, 2G bioethanol can reduce GHG emissions by 86 % compared to gasoline while 1G bioethanol can only decrease emissions by 39 - 52 % (Iram *et al.,* 2022). Amongst biomass varieties, cellulosic feedstocks are believed to have the greatest capacity for alleviating climate change and are extensively available at a lower price per unit energy than petroleum (Lynd, 2017). Furthermore, lignocellulosic biomass (LCB) is far more abundant and less costly than 1G feedstocks (Hu *et al.,* 2008; Timilsina and Shrestha, 2011). The many attributes of 2G biofuels make it an enticing replacement for 1G biofuels, however, the commercial upscaling of cellulosic biofuels is yet to be established worldwide (Lynd, 2017; Timilsina and Shrestha, 2011).

Although cellulosic ethanol offers the most direct course to an inexpensive platform for biofuel production from inedible biomass, it requires innovation to become economically viable (Lynd, 2017). This industry exhibits great processing costs that are linked to the

complexity of its biomass feedstock as well as logistical challenges, i.e., generating, harvesting, and transporting biomass (Balan, 2014). The components of LCB are assembled in a complex that is naturally recalcitrant to enzymatic hydrolysis and as a result, pre-treatment steps are required for its bioconversion into biofuel (Zoghlami and Paës, 2019). Limited progress has been made in the global cellulosic biofuels industry: in 2016, 2G biofuels had a production capacity of 4.4- and 0.7 billion litres for biodiesel and bioethanol, respectively. On the other hand, 1G biofuels had a capacity of 30- and 98-billion litres for biodiesel and bioethanol, respectively, overshadowing the production capacity of 2G biofuels (Lynd, 2017). This was largely due to overly optimistic claims that (i) technologies have been established to make conversions economically viable and (ii) investments were only required for commercialisation and upscaling (Den Haan, 2018; Lynd, 2017). This inefficient progress caused the growth of international biofuel production to level off, policy support to wane, and research and development (R&D) capital to decline and/or narrow in several countries (Lynd, 2017). Market research has been investigating solutions to reduce production costs of 2G biofuels, that will enable it to be cost-competitive with 1G biofuels (Valdivia *et al.,* 2016).

Any efforts to salvage cellulosic biofuels should include investments in innovations that are pursuant to new processing paradigms aimed to address the key economic hindrance of this industry. Such innovations involve upstream technologies designed to manufacture readily processed high-value coproducts from recalcitrant cellulosic biomass (Lynd, 2017). The co-products should be marketed for a high price, thereby reducing the 2G biofuel processing costs (Balan, 2014). As a result, investment in next-generation biofuels is currently more than 50 % in chemicals rather than fuels. Consequently, most biofuel start-up companies that have remained are primarily pursuing a wider range of higher-value bioproducts, thus shifting their focus from biofuels to biorefineries wherein both biofuels and high-value bio-based products are sustainably produced from LCB (Lane *et al.,* 2018a; Lynd, 2017; Shahid *et al.,* 2021). This review primarily aims to investigate the economic and sustainable bioproduction of xylitol, a high-value sugar substitute with a well-established market, intended to improve the process economics of cellulosic biofuels. Additionally, this review will shed light on existing research pertaining to the biorefinery industry along with industrially important products.

1.2 THE BIOREFINERY CONCEPT

Several definitions exist for the term 'biorefinery'; however, the IEA Bioenergy Task 42 provides a thorough explanation: Biorefining is "the sustainable processing of biomass into a spectrum of marketable biobased products and bioenergy/biofuels," (Van Ree and De Jong, 2019). The biorefinery concept encompasses an extensive assortment of technologies able to break down biomass feedstock into their constituents which can be converted to biochemicals, value-added products, and biofuels (Cherubini, 2010). The concept is further discussed below.

1.2.1 The Necessity for the Biorefinery Industry

One of the systemic drivers required to achieve CE is the incorporation of the bioeconomy (BE) (Mohan *et al.,* 2019). A BE is an economy where chemicals, energy, and materials stem from renewable biological feedstocks (Pacheco-Torgal, 2020). It aims to mitigate climate change while supplying replenishable biomass and generating employment and business opportunities. A vital facilitator of the BE is the biorefinery concept whereby biomass conversion is optimised (Ubando *et al.,* 2019).

In accordance with the closed-loop concept of the CE, biorefining represents an essential enabling strategy by 'closing loops' of carbon, minerals, water, and raw biomass materials. Biorefining is, therefore, the best approach for large-scale sustainable use of biomass in the BE. It will lead to the co-production of biobased products, bioenergy, and feed/food ingredients at a competitive cost while optimally impacting the environment and socio-economy (Van Ree and De Jong, 2019). Alternatively, bio-based renewable resources can be more efficiently managed through the concept of a circular bioeconomy (CBE) whereby the principles of CE are integrated into the bioeconomy (Figure 1.2) (D'Amato *et al.,* 2020). The CBE makes use of biorefineries to produce a variety of higher value-added bioproducts and bioenergy from renewable carbon sources via the CE framework (Ubando *et al.,* 2019).

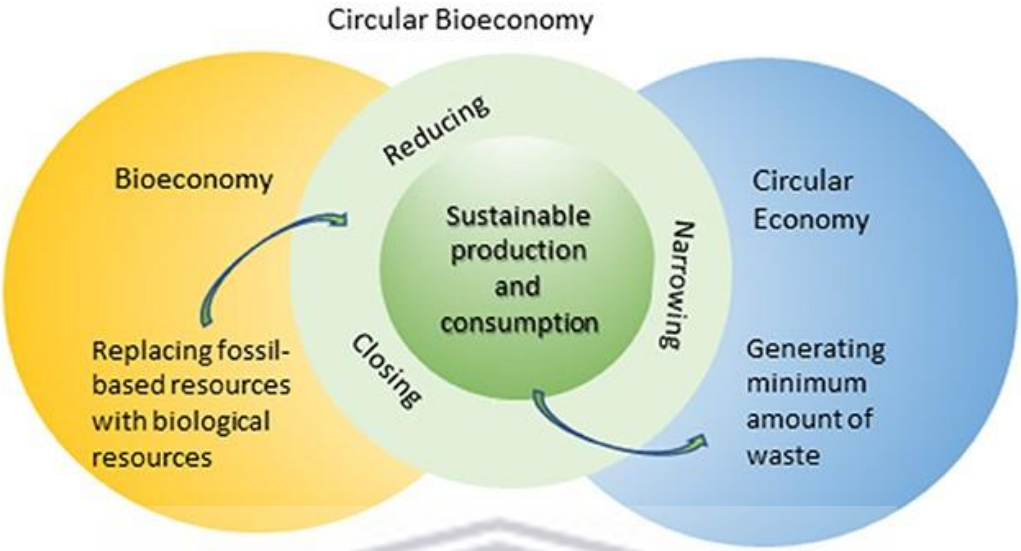

Figure 1.2. Schematic representation of a circular bioeconomy resulting from the intersection of circular economy and bioeconomy concepts (Tan and Lamers, 2021). The closed-loop framework of the circular economy utilises biological resources to sustainably generate products and use goods.

Biorefineries are facilities or networks of facilities that combine biomass conversion methods and equipment, in an optimal manner, to sustainably produce chemicals, power, and transportation biofuels from biomass (De Albuquerque *et al.,* 2019; Cherubini, 2010). Its three main features are (i) the use of diverse feedstock from both unused and residual sources, (ii) the incorporation of several biomass process steps, and (iii) the coupled production of materials (e.g., chemicals) and energy (e.g., biofuels) (Hingsamer and Jungmeier, 2018). The operation intends to be self-sustaining and ecologically sound (Saral *et al.,* 2022). Furthermore, an aim of the industry is to exploit all the synergies for efficient and sustainable production, to maximise the social, environmental, and economic benefits, enabling the progression towards a CBE (Hingsamer and Jungmeier, 2018; Ubando *et al.,* 2019).

1.2.1.1 Evaluating the Sustainability of Biorefineries

Biorefineries ought to be extremely energy efficient and employ generally zero-waste manufacturing processes, whereby 'waste' is considered coproducts to be redistributed for conversion processes or added-value use (Ferreira, 2017). This promise of sustainability is a key incentive for the establishment of the industry (Hingsamer and Jungmeier, 2018). Thus,

the sustainability of the entire value chain of biorefineries must be assessed including the complete life cycle of products from its generation to its employment, and end, including its reprocessing (Figure 1.3). To evaluate the sustainability of a biorefinery, the pillars of sustainability, namely, social, environmental, and economic impacts, must be assessed (Ferreira, 2017).

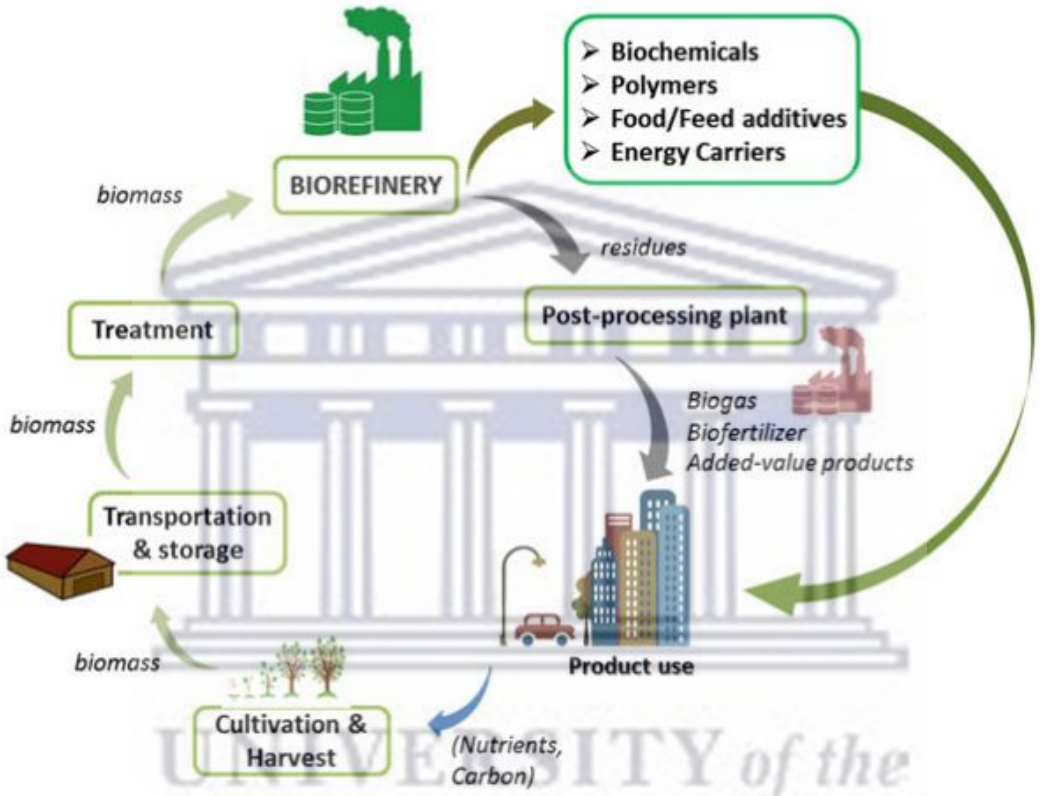

Figure 1.3. Schematic representation of a biorefinery system integrated into the life cycle stages of product development (Ferreira, 2017). A closed loop is depicted from feedstock cultivation and harvest to the final primary product and coproducts developed as well as residues post-processing.

Life Cycle Sustainability Assessment (LCSA) is a method developed to evaluate social, environmental, and economic sustainability (Lin *et al.,* 2020). It represents an aggregate of assessments addressing each dimension of sustainability: Life Cycle Assessment (LCA), Life Cycle Costing (LCC), and Social Life Cycle Assessment (SLCA) for environmental, economic, and social impact evaluation, respectively (Hingsamer and Jungmeier, 2018; Lin *et al.,* 2020; Ubando *et al.,* 2019). All assessments follow the same framework for examination: (i) defining the goal and scope, (ii) life cycle inventory, (iii) life cycle impact (LCI) assessment, and (iv)

interpretation of results (Lin *et al.,* 2020). The succeeding paragraph briefly discusses these assessments.

LCA is a method of evaluating the environmental impact of product production or service delivery throughout its life cycle, from 'cradle-to-grave' (Patrizi *et al.,* 2020; Ubando *et al.,* 2019). LCC is an economic assessment whereby all incurred production costs of a product or service are determined, from the construction of the facility to the expiration of its economic life. This includes all expenses related to resource exploitation, procurement, application, and disposal as well as the risks and externalities (Sasongko and Pertiwi, 2021). SLCA is a method constructed to evaluate the negative and positive social effects of products and services throughout its life cycle. The findings of this assessment aim to better an organisation's social performance and stakeholders' well-being across life cycles (Souza *et al.,* 2021). The employment of LCSA benefits a biorefinery by highlighting negative impacts across the pillars of sustainability and in making decisions aimed at establishing more sustainable products during its life cycle (Wulf *et* al., 2019).

1.2.2 Classifications of Biorefinery Systems

Cherubini *et al.* (2009) described a classification system embraced within the IEA Bioenergy Task 42, wherein all individual biorefinery systems are regarded separately and grouped according to its four main components. The system components used to classify biorefineries are (in order of importance): (i) platforms, (ii) products, (iii) feedstock, and (iv) conversion processes (Cherubini *et al*., 2009; Ghatak, 2011).

1.2.2.1 Platforms

Platforms may be: (i) intermediate products which may be further converted into final products or other intermediates, (ii) connections between separate biorefinery concepts, or (iii) final products (Cherubini *et al*., 2009; Hingsamer and Jungmeier, 2018). The more platforms involved, the more complex the biorefinery system (Hingsamer and Jungmeier, 2018). This system component is the most significant feature in classifying types of

biorefineries, since platforms might be acquired by applying various conversion processes to a range of raw materials (Cherubini *et al*., 2009). Examples of platforms include five-carbon (C5) and six-carbon (C6) sugars, lignin, syngas, and plant-based oils (Philippini *et al.,* 2020). More examples are provided in Table 1.1.

1.2.2.2 Products

Biorefineries generate both energy and non-energy products, and are therefore grouped into two broad categories, namely energy-driven biorefineries and material-driven biorefineries (Cherubini *et al*., 2009; Ghatak, 2011). Bioenergy-based establishments are more prevalent within newly constructed biorefineries. The main products of these biorefineries include biofuels, heat, and power (Table 1.1) while agricultural and process residues are transformed into biobased products with added value such as animal feed, amino acids, pigments, and renewable chemicals (Cherubini *et al*., 2009; Nagappan and Nakkeeran, 2020; Van Ree and De Jong, 2019).

Material-driven, also known as product-driven biorefineries primarily produce higher-value biobased products (Table 1.1) while low-quality agricultural and process residues are used to produce bioenergy and less frequently, biofuels. Such biobased products include chemicals, feed and food ingredients, fertilisers, fibrous materials, and pharmaceuticals. These biorefineries largely occur in the paper and pulp, feed and dairy, and food industries (Cherubini *et al*., 2009; Hingsamer and Jungmeier, 2018; Van Ree and De Jong, 2019). The IEA Bioenergy Task 42 reported that the use of biomass for feed and non-food applications is expected to change from an energy to a material-based approach within the next 10 to 20 years (Van Ree and De Jong, 2019).

1.2.2.3 Feedstocks

Feedstocks are biomass or renewable raw materials that are transformed into profitable products in biorefineries (Cherubini *et al*., 2009). The provision of a consistent, renewable, and regular feedstock supply is an integral component of a biorefinery system (Badjugar and Bhanage, 2018; Cherubini, 2010). Biorefineries need a reliable source of feedstock throughout

its lifespan, for the industry to be a practical replacement for petroleum refineries. Feedstock is the principal driver for a biorefinery blueprint, since 40 % to 60 % of the operating expenses of a standard biorefinery are represented by this system component (Chaturvedi *et al.,* 2020; Ghatak, 2011).

Biorefineries can obtain their feedstock from the following four sectors: (i) agriculture, (ii) forestry, (iii) industrial and domestic activities, and (iv) aquaculture (Cherubini *et al*., 2009). These renewable resources are further distinguished into two subgroups, namely dedicated crops (from agriculture, forestry, and aquaculture) and residues (from industrial, forestry, and agricultural activities) (Table 1.1) (Cherubini *et al*., 2009; Ghatak, 2011). Dedicated feedstocks include starch crops, lignocellulosic crops, sugar crops, oil-based crops, marine biomass, and grasses. Residues are comprised of lignocellulosic residues, oil-based residues, and organic residues (Cherubini *et al*., 2009).

Dedicated crops are a controversial issue as those sourced from agriculture compete with food availability, while aquatic and forestry dedicated crops are in contention with food production for resources such as water and land (Ghatak, 2011). Cultivating these crops on ground transformed from naturally vegetated lands results in more carbon dioxide (CO_2) released into the atmosphere than is saved by the biorefinery concept (Fargione *et al.,* 2008). On the other hand, all residue feedstocks are not in competition with food availability or production. Agricultural residues are extensively accessible at relatively lower prices internationally (Ghatak, 2011). In 2008, Gabrielle and Gagnaire estimated that 10^{10} Mt (megatonnes) of agricultural residues were available globally, corresponding to 47 EJ (exajoules) of energy. However, the benefits and detriments of utilizing this biomass in biorefineries are being debated (Cherubini and Ulgiati, 2010).

Collecting these residues from fields induces adverse repercussions in a process known as land-use change (LUC) effects. Such effects include soil erosion, soil organic matter turnover, decrease in crop yields, and change in nitrous oxide (N_2O) soil emissions. (Cherubini and Ulgiati, 2010; Ghatak, 2011). Nonetheless, considering all aspects, including LUC effects, GHG emissions and non-renewable energy demands are reduced by biorefineries utilising crop residues. Exploiting one tonne of agricultural residue in a biorefinery system saves 0.26 – 0.33

tonnes CO_2-eq./t, while the bioproducts' energy content comprises 4 – 5 times the non-renewable energy input when compared to its fossil reference system (Cherubini and Ulgiati, 2010). Among the assortment of biomass, lignocelluloses have been extensively researched for utilization in biorefineries due to its promising applications to greatly capitalize the feedstock into a range of bio-based products. (Ashokkumar *et al.,* 2022; Chaturvedi *et al.,* 2020). Being the most abundant biomass around the globe, lignocelluloses are sourced from various sectors including agricultural, forestry, and industrial residues, organic municipal solid waste, and dedicated crops (Ashokkumar *et al.,* 2022; Dhamen *et al.,* 2018).

1.2.2.4 Processes

Researchers review biorefineries according to the possible conversion processes implemented on specific feedstock, since biomass can be processed in several ways depending on the desired product (Chaturvedi *et al.,* 2020). Conversion processes in biorefineries aim to depolymerize and deoxygenate the feedstock to transform it into value-added products (Cherubini, 2010). Many literary sources agree that the four major technological processes included in a biorefinery system are: (i) mechanical, (ii) chemical, (iii) biochemical/biotechnological, and (iv) thermochemical (Cherubini *et al*., 2009; Gavrilescu, 2014; Hingsamer and Jungmeier, 2018; Parajuli *et al.,* 2015; Ubando *et al.,* 2019).

Biomass processing typically begins with mechanical conversion whereby feedstocks are reduced in size, or its components are separated to provide a substantial surface area, making the conversion process efficient (Chaturvedi *et al.,* 2020). Mechanical processes include distillation, milling, and pre-treatment (Cherubini *et al*., 2009). Chemical processes change the chemical properties of biomass in the presence of a catalyst. Enzymatic hydrolysis and transesterification are the most common chemical conversion processes utilised by biorefineries (Chaturvedi *et al.,* 2020). Biochemical/biotechnological processes utilise enzymes or microorganisms to convert carbohydrates into various products such as sugars, acids, and alcohols (Chen and Wang, 2017; Cherubini *et al*., 2009; Gavrilescu, 2014). The most frequently used biotechnological processes are anaerobic digestion, enzymatic hydrolysis, and fermentation (Chaturvedi *et al.,* 2020). Thermochemical processes subject biomass to extremely high temperatures and/or pressures in the absence or presence of a catalyst

(Cherubini *et al*., 2009). There are three main thermochemical processes utilised by biorefinery systems, namely, combustion, gasification, and pyrolysis (Ferreira, 2017). The thermochemical conversion of biomass generates bioenergy products and biochemical building blocks (Jha *et al.,* 2022; Tanger *et al.,* 2013). Usually, various conversion processes are employed subsequently, or in parallel to reach the desired product(s) (Chaturvedi *et al.,* 2020).

All these technological processes require additional energy and material inputs. The end objective is to replace these auxiliary energies and materials with renewable sources such as solar power and process residues. This is already being achieved in lignocellulosic biorefineries producing bioethanol: the electricity and heat needed by the system are sourced from the combustion of lignin (the process residue) (Cherubini *et al*., 2009). The features and respective subgroups of the biorefinery system are summarised in Table 1.1 below. Examples of each system component are included.

Table 1.1. The four features used to classify a biorefinery system, including some examples. Adapted from (Cherubini et al., 2009).

Platforms	Products	Feedstocks	Processes
C5 sugars	**Energy**	**Dedicated crops**	**Thermochemical**
C6 sugars	Biodiesel	Oil crops	Combustion
Oils	Bioethanol	Sugar crops	Gasification
Biogas	Biomethane	Starch crops	Pyrolysis
Syngas	Synthetic biofuels	Lignocellulosic crops	
Hydrogen	Electricity and heat	Grasses	**Biochemical**
Organic juice		Marine biomass	Fermentation
Pyrolytic liquid	**Material**		Anaerobic digestion
Lignin	Food	**Residues**	Aerobic conversion
Electricity and heat	Animal feed	Lignocellulosic residues	Enzymatic processes
	Fertilizer	Oil-based residues	
	Glycerine	Organic residues	**Chemical**
	Biomaterials	Others	Enzymatic hydrolysis
	Chemicals and building blocks		Hydrogenation
	Polymers and resins		Transesterification
	Biohydrogen		Pulping
			Mechanical
			Extraction
			Distillation
			Milling
			Pre-treatment

1.2.3 Motivations for Integrated Biorefineries

Driven by the increased demand for biofuels in the transportation sector, biorefineries require the development of advanced bioenergy production processes to make it cost-competitive with fossil fuel energies (Cherubini *et al.,* 2009; Budzianowski, 2017). Biomass may be advantageously employed in numerous practical applications due to its high diversity and its unique chemical composition that cannot easily be artificially replicated. Therefore, the conversion of biomass to only simple bioenergies (biofuels, bioheat, and biopower) results in a great loss of biomass feedstock potential. To optimise biomass valorisation and improve the economics of biorefineries, cascading strategies are required (Budzianowski, 2017). Case studies analysed by Budzianowski (2017), revealed that biorefineries that coproduce high-value bioproducts and high-yield bioenergies, achieve large profit margins. Coproduction of bioenergies and bioproducts may be achieved by integrating current biofuel plants with novel bioindustries. This integration has the potential to enhance the development of bioenergy-related businesses and is necessary for realising the economic feasibility of industries within the CBE (Budzianowski, 2017; Ubando *et al.,* 2019).

Bioenergies are produced in high volumes but are low in value (due to current fossil fuel prices) while bioproducts are produced in low volumes but are high in value (Budzianowski, 2017; Diaz-Chavez *et al*., 2016; Hingsamer and Jungmeier, 2018). These bioproducts are of low volume because they either exist in low concentrations in feedstock or are acquired via highly developed conversion routes producing significant amounts of by-products. Examples of bioproducts include (in order of decreasing value): (i) biopharmaceuticals, (ii) biocosmetics, (iii) bionutrients, (iv) biochemicals, (v) biofertilisers, and (vi) biomaterials. Figure 1.4 schematically illustrates the relative values and volumes of these six bioproducts and various bioenergies produced in biorefineries. The profitability of biorefineries is intended to be increased by high-value bioproducts while high-volume bioenergies generated on-site will reduce energy expenses for internal use and provide additional income (Budzianowski, 2017).

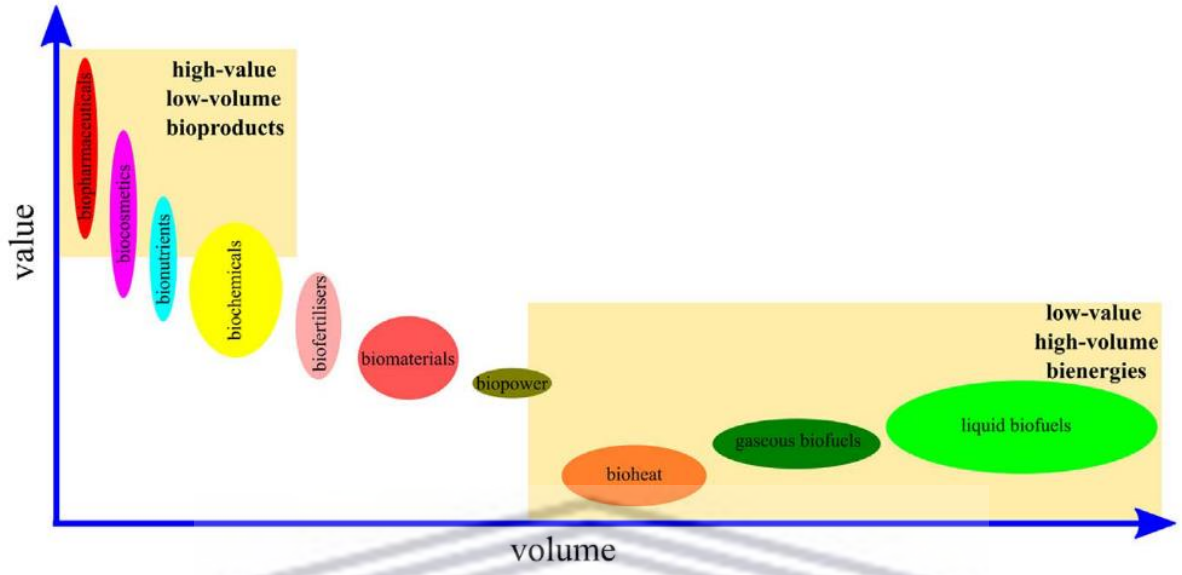

Figure 1.4. Schematic illustration of relative values and volumes of a variety of bioproducts and bioenergies produced from biorefineries (Budzianowski, 2017). The left half of the graph depicts the bioproducts, in order of decreasing value: biopharmaceuticals, biocosmetics, bionutrients, biochemicals, biofertilizers, and biomaterials. The high-volume bioenergies are depicted on the right half of the graph in order of increasing volume: biopower, bioheat, gaseous biofuels, and liquid biofuels.

It is believed that, globally, renewable biomass could be used to produce over 90 % of petroleum products, and that by the year 2030, 50 % of the pharmaceutical- and 33.33 % of the chemical market could be biobased (Clauser *et al.,* 2021). High-value bioproducts, however, need to be assessed to determine their suitability for biorefineries. The two essential requirements for biorefinery-based bioproducts are: (i) a great degree of coupled production of bioenergies and bioproducts and (ii) the viability of large-scale production (Budzianowski, 2017). The six groups of bioproducts are briefly discussed below.

1.2.3.1 High-value, low-volume bioproducts

1.2.3.1.1 Biopharmaceuticals

The biopharmaceutical industry is appraised at more than $ 140 billion (Taunt *et al.,* 2018). Certain plant species contain relatively high concentrations of natural substances with pharmaceutical properties. Satisfactorily high concentrations decrease separation costs enabling the production of highly pure biopharmaceuticals at adequate production costs. The

exploitation of biopharmaceuticals requires the utilization of specific natural substances readily available in raw biomass since they are mainly obtained via separation processes. Biopharmaceuticals suited for biorefinery processes and bioenergy integration include algal pharmaceuticals, extracts, and vitamins (Budzianowski, 2017).

Algae are an abundant source of biopharmaceuticals due to their high biochemical diversity (Salami *et al.,* 2021). Algal pharmaceuticals include anticancer, antifungal, antimicrobial and antiviral compounds (Budzianowski, 2017; Salami *et al.,* 2021). Once high value bioproducts have been extracted from algal biomass, the residual feedstock can be processed into lipids and proteins to be used as biofuels or refined for bioenergy harvesting. Plants can be used to directly produce biopharmaceuticals - where crops serve as drug-producing bioreactors - which are then extracted for enrichment (Budzianowski, 2017). Extracts include alpha-galactosidase from tobacco, gastric lipase from corn and human growth hormone obtained from potato (Elbehri, 2005). Microbial biomass may also be sources of biopharmaceutical extractions. Vitamins synthesised by fungi or yeast result in reduced production costs, pollutants and waste compared to artificially produced vitamins since only a single-integrated step is required for the generation of the former. Biobased vitamins may be created in biorefineries via extraction from plants (vitamin E and D3) or biotechnological procedures (vitamin C and B2) (Budzianowski, 2017).

1.2.3.1.2 Biocosmetics

The global biocosmetic market is predicted to reach approximately $ 25 billion by 2024 (Goyal and Jerold, 2021). Biocosmetics may be developed from fats and vegetable oils and bioenergy may be harvested from the residual biomass. One biomass of interest is cardoon. Extracted cardoon oil is converted to high-value azelaic and pelargonic acids which serve as building blocks for valuable biocosmetics (Budzianowski, 2017).

1.2.3.1.3 Bionutrients

The feed and food industry can be coupled with bioenergy production since the former generates biowastes (Tsegaye *et al.,* 2021). Bionutrients need to be highly pure as they should be free of harmful ingredients. Consequently, sophisticated conversion processes are required to produce bionutrients. Fortunately, the bioenergy production process is feasible and has the potential to improve the finances of bionutrient-producing biorefineries. Examples of bionutrients include algal nutrients, feed or food additives, proteins, and speciality nutrients (Budzianowski, 2017).

Algae-based bionutrients include polysaccharides, omega-3 fatty acids and carotenoids (Barkia *et al.,* 2019; Budzianowski, 2017). Feed or food additives applied in the production of various bionutrients consist of xylitol, xylanase, water-insoluble fibres, protein hydrolysates, and pectin-based additives (Budzianowski, 2017; Kamat *et al.,* 2013; Laohakkunjit *et al.,* 2014). Proteins derived from biomass serve as promising alternatives to animal proteins while bioenergy can be more efficiently produced from residues (Budzianowski, 2017). Amino acids and proteins can be coproduced by vegetable oil- and biofuel-producing biorefineries. Amino acids can then be further processed into chemicals (Scott *et al*., 2014). Astaxanthin is an example of a speciality nutrient, more specifically a nutraceutical, that has applications in fish farming (Budzianowski, 2017).

1.2.3.1.4 Biochemicals

Biochemicals, according to Budzianowski (2017), represent the largest group of high value bioproducts suited for biorefineries. However, biochemical production is restricted by underdeveloped conversion technologies. In 2010, the US Department of Energy (DOE) identified the top ten biochemicals produced from biorefinery carbohydrates that had notable market potential namely (in alphabetical order): biohydrocarbons, ethanol, furans, glycerol and derivatives, hydroxypropionic acid/aldehyde, lactic acid, levulinic acid, sorbitol, succinic acid and xylitol (Bozell and Petersen, 2010). Biochemicals can be further categorised into algal chemicals, building blocks, extracts and specialty chemicals, and intermediates (Budzianowski, 2017).

Numerous biochemical-based building blocks have proven niche markets (Millán *et al.,* 2019). Such building blocks include biosyngas, citric acid, glycerol, proline, sorbitol, and xylitol. Within biorefineries, most building blocks are produced via conversion processes coupled to bioenergies. Lower-value biomass are potentially rich sources of extracts such as steroidal compounds, tannins, and waxes (Budzianowski, 2017). Industrial enzymes are sourced from low-cost food wastes (Sharma *et al*., 2022). After high value biochemicals are extracted via cascading approaches, the residues serve as bioenergy feedstock (Budzianowski, 2017). Biorefinery operations increase in value when biochemical building blocks are further converted to secondary biochemicals. Biodiesel and bioethanol derivatives also serve as high-value intermediate biochemicals which enhance the sustainability and economics of standard biorefineries (Posada *et al.,* 2013; Yan *et al.,* 2014).

1.2.3.1.5 Biofertilisers

The CE stipulates that fertiliser be produced in a closed loop where residual biomass is re-integrated into the environment (Chojnacka *et al.,* 2020; Budzianowski, 2017). Biofertilisers that are generated *in situ* and comprise low-calorific residual feedstock with minute bioenergy, have the potential to supplant chemical fertilisers. Various biofertilizers are generated as by-products of bioenergy production (Budzianowski, 2017). Such fertilisers include ash from biomass combustion or gasification, digestate from anaerobic digestion, and residual biomass from bioethanol production (Budzianowski, 2017; Bušić *et al.,* 2018).

1.2.3.1.6 Biomaterials

The conversion of biomass to biomaterials generates biowaste that can be further processed into bioenergy leading to strong, coupled production processes. Due to the complexity of biomass structures, biomaterials cannot easily be artificially replicated from fossil fuel feedstocks. As such, several biomaterials can be produced at rates cost-competitive with fossil fuel-derived materials (Budzianowski, 2017). There is a substantial market for biomaterials, making them suitable biorefinery bioproducts (Budzianowski, 2017; Leong *et*

al., 2021). This large class of bioproducts include adhesives, fibres, packaging materials, plastics, textiles and vanillins (Ghatak, 2011; Tong *et al.,* 2021).

1.3 LIGNOCELLULOSIC BIOMASS

In accordance with the principles of the CBE, biomass is regarded as a renewable resource to be used as feedstock to produce energy and chemicals (Takkellapati *et al*., 2018). Furthermore, LCB has been identified as the most abundant and widespread renewable resource to be exploited to produce value-added products and biofuels (Singhania *et al*., 2022; Takkellapati *et al*., 2018). Annually, approximately 181.5 billion tonnes of this inexpensive resource is produced (Dhamen *et al.,* 2018; Ge *et al*., 2018; Yousuf *et al*., 2020). The large-scale exploitation of LCB in biorefineries is significant for the progression toward a CBE (Banu *et al.,* 2021; Singhania *et al*., 2022). To understand the recalcitrance of this substrate and the challenges in making cost-effective products from it, we will subsequently explore LCB structure and conversion processes.

1.3.1 Lignocellulose Composition

Lignocelluloses are predominantly comprised of two polysaccharides, cellulose and hemicellulose, and the phenolic polymer, lignin (Abdel-Hamid *et al.,* 2013; Zoghlami and Paës, 2019). The percentage composition of these three constituents varies across different sources of LCB, however, the average compositions are in the ranges of 40 – 50 % cellulose, 25 – 30 % hemicellulose, and 15 – 20 % lignin (Alonso *et al*., 2010; Rezania *et al.,* 2017).

Cellulose is a homogenous polymer composed of D-glucose subunits (7 000 to 15 000 units) joined by β-1,4-glycosidic bonds (Brethauer and Studer, 2015). Multiple layers of linear cellulose molecules overlap and are stabilised via intra- and inter-molecular hydrogen bonds forming amphiphilic microfibrils, making cellulose polymers stable and insoluble (Woiciechowski *et al*., 2020). These attributes make it challenging to hydrolyse cellulose to its constituent D-glucose monomer (Takkellapati *et al*., 2018). Hemicellulose is an amorphous, heteropolymer composed of various five-carbon (e.g., arabinose, rhamnose, and xylose) and six-carbon (e.g., galactose, glucose, and mannose) sugars. The distribution of these

monomeric sugars differs dramatically based on the nature of the biomass (Li *et al*., 2013a; Takkellapati *et al*., 2018). For example, the hemicellulose of crop residues and hardwoods are mainly comprised of xylan, a xylose polymer, whereas glucose and mannose are the major monomeric sugars in the hemicellulose of softwoods (Takkellapati *et al*., 2018). Unlike cellulose, hemicellulose has little inherent chemical resistance and physical strength, therefore it can readily undergo hydrolysis (Li *et al*., 2013a; Takkellapati *et al*., 2018).

Lignin is an amorphous phenolic polymer comprised of three main crosslinked monolignols: sinapyl alcohol, *p*-coumaryl alcohol, and coniferyl alcohol (Abhilash and Thomas, 2017; Patil *et al*., 2016; Zhou *et al.,* 2016). This polymer is responsible for the structural integrity of cell walls as it joins hemicelluloses to cellulose in plant cell walls (Figure 1.5) (Zeng *et al.,* 2017; Zoghlami and Paës, 2019). Lignin is hydrophobic and structurally rigid, and as such it physically obstructs enzyme accessibility to cellulose and irreversibly adsorbs enzymes amid enzyme hydrolysis (Zoghlami and Paës, 2019). The chemical structures of cellulose, hemicellulose, and lignin as well as the interactions between them render LCB recalcitrant to enzymatic and microbial decomposition (Brethauer and Studer, 2015; Woiciechowski *et al*., 2020).

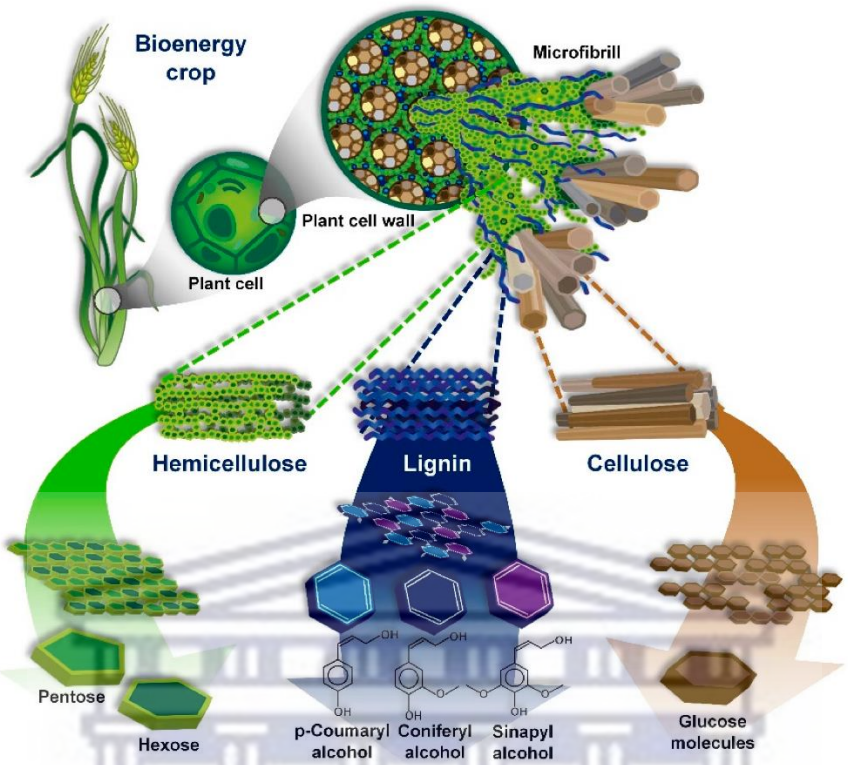

Figure 1.5. Lignocellulosic biomass structure and its three main constituents: cellulose, hemicellulose, and lignin (Hernández-Beltrán *et al.,* 2019). Lignin joins hemicellulose to cellulose thereby forming a lignin-covered matrix that protects both polysaccharide components.

1.3.2 Lignocellulose Conversion to Value-Added Products

Lignocellulosic biomass conversion to fuels and chemicals occurs via two distinct processes: thermochemical or biochemical conversion (Tanger *et al.,* 2013). To reiterate: thermochemical processes subject biomass to extremely high temperatures and/or pressures in the absence or presence of a catalyst (Cherubini *et al*., 2009). These conversion processes generate bioenergy products and biochemical building blocks (Jha *et al.,* 2022; Tanger *et al.,* 2013). Biochemical processes utilise enzymes or microorganisms to convert carbohydrates to various products such as sugars, acids, and alcohols (Chen and Wang, 2017; Cherubini *et al*., 2009; Gavrilescu, 2014).

Of the two conversion pathways, thermochemical reactions occur at faster rates, owing to the high temperature and pressure, and catalyst inputs (Jha *et al.,* 2022). However, biochemical processes are more economically feasible and environmentally friendly due to

superior conversion efficiencies and gentler operating conditions. As a result, enzyme-based biochemical processes are deemed the most promising of biomass conversion technologies (Mohite and Patil, 2016).

The bioconversion of LCB is achieved via three steps, namely (i) pretreatment, (ii) enzymatic hydrolysis/saccharification, and (iii) fermentation (Li *et al.,* 2023; Sankaran *et al.,* 2021). The recalcitrant nature of LCB requires that it be pretreated (physically or chemically) before being subjected to processing technologies to make the holocellulose (hemicellulose and cellulose) available for biochemical conversion by enzymes or microorganisms (Devi *et al.,* 2022; Hernández-Beltrán *et al.,* 2019; Li *et al.,* 2023). A side effect of this step is the formation of pretreatment-derived inhibitors that repress downstream biochemical processes, namely, microbial fermentation, growth, and metabolism (Jönsson and Martín, 2016; Todhanakasem *et al.,* 2018). Consequently, the use of microorganisms resistant to these toxic inhibitors is essential (Hasunuma *et al.,* 2013). The second step of LCB bioconversion hydrolyzes cellulose and hemicellulose into fermentable sugars by means of costly hydrolytic enzymes (Den Haan, 2018; Huang *et al.,* 2011; Sankaran *et al*., 2021). During the final step, the monomeric sugars (C5 and C6) are fermented into desired products (Huang *et al.,* 2011; Sankaran *et al*., 2021). Table 1.2 lists examples of value-added products derived from the different LCB components, their derived products, and production technologies.

Table 1.2. Biorefinery products sourced from the three main components of lignocellulosic biomass along with their derived products and conversion processes. Adapted from (Clauser *et al.,* 2021).

Products	Derived products	Conversion processes	References
Hemicellulose			
Xylitol	Pharmaceutical and food products	Xylose reduction	(Umai *et al.,* 2022)
Levulinic acid	Fuel additives and polymers	Hexose acid catalysis	(Bozell *et al.,* 2000)
Formic acid	Textile, pharmaceutical, and chemical products	Hexose and xylose acid catalysis	(Bulushev and Ross, 2018)
Furfural	Fuels, fuel additives, and chemicals	Xylose acid catalysis	(Binder *et al.,* 2010)
Cellulose			
Ethanol	Mainly biofuels	Glucose fermentation	(Tse *et al.,* 2021)
Lactic acid	Pharmaceutical and food products	Glucose fermentation	(Abedi and Hashemi, 2020)
Nanocellulose	Polymer additives, papers, and paints	Oxidation and microfibrillation	(Nalintip *et al.,* 2021; Phanthong *et al.,* 2018)
Sorbitol	Pharmaceutical and food products	Glucose fermentation	(Marques *et al.,* 2016)
Lignin			
Vanillin	Food and chemical products	Oxidation	(Tobias *et al.,* 2019)
Lignosulfonates	Chemical products and adhesive additives	Hydrolysis and sulfonation	(Aro and Fatehi, 2017)

Commercially, the steps of biomass conversion are achieved through separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF) (Lynd *et al.,* 2017). The former involves enzyme saccharification of pretreated lignocellulose at a

temperature optimised for the saccharifying enzyme. Thereafter suitable microorganisms are added to ferment the hydrolysate. The SSF process occurs in the same reactor where enzymatic hydrolysis and fermentation are simultaneously performed (Ishikazi and Hasumi, 2014). Despite these process developments, the addition of costly hydrolytic enzymes severely affects the cost-effectiveness of lignocellulosic biorefineries. A desirable strategy to improve process economics is the consolidation of saccharification and fermentation methodologies in a process termed consolidated bioprocessing (CBP) (Den Haan, 2018; Hasunuma *et al.,* 2013). Consolidated bioprocessing is the most progressive processing option whereby genetically engineered microorganisms or artificial co-cultures accomplish the lignocellulosic bioconversion in a single-step process (Brethauer and Studer, 2015). This single-step conversion process has the potential to produce value-added products at a low cost since the large expenses incurred by the production of microbial enzymes can be avoided (Hasunuma *et al.,* 2013).

1.4 XYLITOL: APPLICATIONS, HEALTH BENEFITS, AND BIOPRODUCTION

As described above, the objective of a LCB biorefinery is the conversion of feedstock into an extensive range of value-added chemicals and/or fuels (Hernández-Pérez *et al*., 2019). One such value-added chemical is xylitol (Rao *et al.,* 2016). Xylitol is produced from xylose, the second most abundant sugar found in nature, and can aid in enabling the economic viability of lignocellulosic biorefineries (de Albuquerque *et al.,* 2014; Narisetty *et al.,* 2022). Xylitol has diverse applications across a range of industries, which has driven its market growth and rendered it one of the top ten value-added biochemicals (Ahuja *et al.,* 2020; Budzianowski, 2017; Hernández-Pérez *et al*., 2019). Annually, 200 000 tonnes of xylitol are produced worldwide, and with a compound annual growth rate (CAGR) of 5.8 % between 2021 and 2026, its global market is expected to be valued at US$ 1 billion by 2026 (Ravella *et al.,* 2022). This value-added chemical has a Generally Regarded as Safe (GRAS) status and as such, is utilised in the cosmetics, food, odontological, pharmaceutical, and polymer industries (Ahuja *et al.,* 2020; Benahmed *et al.,* 2020; Lugani and Sooch, 2017).

Xylitol ($C_5H_{12}O_5$) is a white crystalline, five-carbon sugar alcohol that is highly soluble (De Albuquerque *et al.,* 2014; Umai *et al.,* 2022). Commercially, it is used as a sugar alternative

since xylitol has a sweetness potency almost equal to sucrose but with 40 % fewer calories. The effectiveness of sugar substitutes, such as aspartame, erythritol, saccharine, sorbitol, stevia, and xylitol, is determined by considering their glycaemic index (GI), calories per gram (cal/g), and sweetness potency (Kumar *et al.,* 2022). Although xylitol has the highest GI of these substitutes (GI of 7), it is still considerably low (Burgess, 2019; Kumar *et al.,* 2022). Aspartame has a caloric value of 4 cal/g whereas xylitol only provides 2.4 cal/g (Chattopadhyay *et al.,* 2014). Side effects of xylitol consumption include bloating, diarrhea, and stomach aches whereas health risks associated with the use of other alternate sweeteners include acute toxicity, increased blood pressure, inflammations, methanol toxicity, nausea, obesity, and tooth decay (Kumar *et al.,* 2022). Due to these attributes, xylitol is considered an effective sugar substitute, making it one of the leading commercially manufactured sugar alcohols (Umai *et al.,* 2022).

Trace amounts of xylitol occur naturally in diverse fruits and vegetables, hardwood trees, and plant stalks and husks (Chen *et al.,* 2010; Umai *et al.,* 2022). Xylitol is also produced in small quantities during glucose metabolism in humans and animals. Given the low quantities of naturally occurring xylitol, its extraction is inefficient (Umai *et al.,* 2022). Currently, large-scale production is achieved by the chemical reduction of xylose in the presence of a nickel catalyst. However, the drawbacks of this technique include costly production, high energy consumption, and extensive non-eco-friendly purification procedures (De Albuquerque *et al.,* 2014; Rao *et al.,* 2016; Takkellapati *et al.,* 2018; Ur-Rehman *et al.,* 2015). Since global xylitol demand is increasing, steep rises in its production have occurred which has prompted researchers to investigate alternate manufacturing processes (Ahuja *et al.,* 2020; de Albuquerque *et al.,* 2014). Particular attention has been paid to the biotechnological route where microorganisms reduce xylose to xylitol by the enzyme xylose reductase (de Albuquerque *et al.,* 2014; Ishikazi and Hasumi, 2014). This process is appealing in the context of a biorefinery as it offers a more economic and environmentally friendly approach to adding value to the xylose found in hemicellulose hydrolysates (Carneiro *et al.,* 2019; Ur-Rehman *et al.,* 2015).

1.4.1 Xylitol Applications and Health Benefits

Xylitol is commonly used as a sweetener in non-cariogenic confectioneries and oral hygiene and pharmaceutical products (Benahmed *et al.,* 2020; Gupta, 2018; Nayak *et al.,* 2014).

1.4.1.1 Xylitol in the food industry

The demand for xylitol is growing as consumers are inclined towards food products that are low in calories and free of sugar due to weight and health awareness (Rao *et al.,* 2016). Approximately 70 % of produced xylitol is used in the manufacturing of chewing gums and confectioneries (Umai *et al.,* 2022). The food industry uses xylitol to improve the colour, shelf-life, taste, and texture of products (Kumar *et al.,* 2022; Umai *et al.,* 2022). Replacing sucrose with xylitol in food products aids in stabilising glycemia levels and reducing general lipid storage which contributes to weight loss and indirectly reduces the risk of cardiovascular problems. It is safe for consumption by diabetic individuals as xylose metabolism is independent of insulin. When consumed it is digested into water and CO_2 (Umai *et al.,* 2022).

1.4.1.2 Xylitol promotes good oral hygiene

Sugar-free chewing gum represents the world's foremost application of xylitol (Ur-Rehman *et al.,* 2015). The dental benefits of xylitol-based oral hygiene products (chewing gum, toothpaste, mouthwash) include anti-cariogenic (it can reduce cavities by up to 100 %), anti-gingivitic and remineralising properties. Oral cavity microflora creates dental caries by fermenting sugars, from consumed food, into acid that demineralises tooth enamel. These bacteria cannot metabolise xylitol, which enables this sugar alcohol to have an anti-plaque effect on teeth, providing xylitol with anti-cariogenic potential (Benahmed *et al.,* 2020). The routine consumption of xylitol has been shown to directly inhibit the growth of *Streptococcus mutans* in dental plaque by starving the microorganism (Benahmed *et al.,* 2020; Gupta, 2018; Nayak *et al.,* 2014). Xylitol-based chewing gums are reported to (i) promote the uptake of calcium phosphate used to remineralise tooth enamel and (ii) reduce gum inflammation (gingivitis) (Benahmed *et al.,* 2020; Nayak *et al.,* 2014).

1.4.1.3 The pharmaceutical/medicinal applications of xylitol

Owing to xylitol's anti-cariogenic and -microbial properties, and suitability for diabetics, it is used to sweeten vitamin formulations, tonics, and cough syrups (Feigal *et al.,* 1981). Xylitol will not react with active ingredients in pharmaceuticals or undergo Maillard reactions since it is chemically inert (Ur-Rehman *et al.,* 2015). The anti-bacterial and anti-inflammatory potential of xylitol enables it to treat and prevent several diseases such as pneumonia, sinusitis, and middle ear and respiratory tract infections (Benahmed *et al.,* 2020; Salli *et al.,* 2019). This non-digestible carbohydrate enters the colon where it is fermented by saccharolytic microflora to generate by-products such as short-chain fatty acids (SCFAs), gases, organic acids, and ethanol. Therefore, xylitol is said to be '*an emerging prebiotic'* since SCFAs maintain cholesterol levels, reduce the risks of irritable bowel syndrome, improve the immune response, and control gut integrity (Lugani and Sooch, 2017; Ur-Rehman *et al.,* 2015). The indigestible but fermentable attribute of xylitol also assists in relieving constipation and improving bone mineral density. Additionally, xylitol improves the barrier function in the skin and inhibits the growth of possible skin pathogens (Salli *et al.,* 2019).

1.4.2 Bioproduction of Xylitol

One of the main bottlenecks in the economic feasibility of LCB-based biorefineries is the neglect of xylose valorisation due to the favourable utilisation of glucose and/or the lack of xylose metabolism in microbial systems, particularly in mixed sugar feedstocks (Chandel *et al.,* 2018; Narisetty *et al.,* 2022). To address this shortcoming, the biotechnological production of xylitol is focused on the xylose metabolism of microorganisms that can naturally assimilate pentose sugars (Narisetty *et al.,* 2022). Numerous engineered and natural strains are studied for increased xylitol production (Umai *et al.,* 2022).

1.4.2.1 Xylose metabolism

It is imperative to understand the regulatory systems of xylose metabolism, to enhance xylose assimilation in organisms (Jin *et al.,* 2004; Zheng *et al.,* 2020). Xylose can only be used and

metabolised in the form of xylulose. Phosphorylated xylulose (xylulose 5-phosphate) then enters the pentose phosphate pathway (PPP) where it is metabolised (De Albuquerque *et al.,* 2014). Microorganisms accomplish the conversion of xylose to xylulose via two principal pathways: the isomerase and the oxidoreductase pathway (Figure 1.6) (Kwak and Jin, 2017).

The isomerase pathway is generally employed by prokaryotes where xylose is isomerized into xylulose in a single enzymatic reaction by means of xylose isomerase (XI, EC 5.3.1.5). (Domingues *et al.,* 2021; Kwak and Jin, 2017). Eukaryotic microorganisms (such as filamentous fungi and yeast) typically convert xylose to xylulose via the two-step oxidoreductase pathway: NADPH-dependent xylose reductase (XR, EC 1.1.1.30) first reduces xylose to xylitol, which is then oxidized by NAD^+-dependent xylitol dehydrogenase (XDH, EC 1.1.1.9) into xylulose (de Albuquerque *et al.,* 2014; Domingues *et al.,* 2021; Kwak and Jin, 2017). Xylitol is therefore an intermediate of the oxidoreductase pathway. It can be secreted out of cells or oxidized by XDH depending on the cofactor availability; to limit the oxidation of xylitol to xylulose, a continuous supply of NADPH is required (Hernández-Pérez *et al*., 2019; Narisetty *et al.,* 2022).

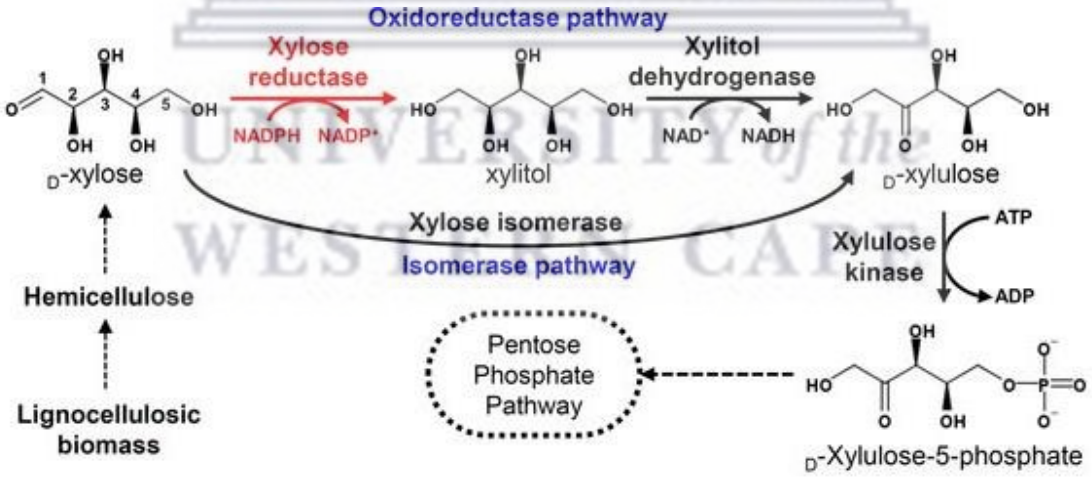

Figure 1.6. The two principal pathways of xylose conversion to xylulose (oxidoreductase and isomerase pathways) before it enters the pentose phosphate pathway. Adapted from (Son *et al.,* 2018). Xylitol is an intermediate metabolite of the oxidoreductase pathway produced by the reduction of xylose by the NADPH-dependent enzyme xylose reductase.

Microorganisms utilising the oxidoreductase pathway may be beneficial for the generation of non-ethanol products or may be used to exploit the cofactor imbalance to drive desirable

reactions (Lane *et al.,* 2018a). The imbalance between NADPH and NAD^+ can drive the inefficient assimilation of xylose and increased production of xylitol. Intriguingly, xylose metabolism in engineered *Saccharomyces cerevisiae* strains exhibits a respiratory response (when consumed it is digested into water and CO_2) unlike the fermentative metabolism displayed by glucose consumption. This suggests that the resultant carbon metabolism may be susceptible to perturbation and able to bypass the metabolic barriers applied by glucose repression (Kwak and Jin, 2017; Lane *et al.,* 2018a).

1.4.2.2 Engineering Saccharomyces cerevisiae to produce xylitol from hemicellulose hydrolysates

Much research has gone into screening microbial strains that can produce xylitol efficiently (Hernández-Pérez *et al*., 2019; Narisetty *et al.,* 2022). Some earlier studies performed in the 1970s revealed that few bacterial strains could produce xylitol. Research performed to determine the xylitol yields of bacteria, filamentous fungi, and yeast have shown that the latter favours xylitol production the most. Therefore, yeast is extensively researched because of its high xylitol yield and xylose assimilation (Umai *et al.,* 2022). *Candida* species are known to be the best natural xylitol producers since they have the highest XR activity, obtaining a yield of 0.84 g xylitol per g xylose with a productivity of 1.01 g/L h^{-1} (Carneiro *et al.,* 2019; Umai *et al.,* 2022). Unfortunately, there are drawbacks to utilising these species for xylitol production. *Candida* sp. assimilate xylose for metabolism and cell growth which lowers the amount of xylitol that can be accumulated (He *et al.,* 2021; Oh *et al.,* 2013). They are also opportunistic pathogens and lack a GRAS status (Carneiro *et al.,* 2019). In light of this, a potentially better species for xylitol production is the industrial yeast strain *S. cerevisiae* (Oh *et al.,* 2013).

Although *S. cerevisiae* is not a native xylose-assimilating yeast, it can be easily and economically genetically engineered to produce xylitol since it has a highly adjustable DNA transformation system and its entire genome is sequenced (Oh *et al.,* 2013; Sherman, 2002; Stewart, 2014). Additionally, it displays high tolerance to inhibitor compounds released in hemicellulose hydrolysates and has a negligible ability to further metabolise xylitol via XDH. While some strains of *S. cerevisiae* possess genes that encode proteins with XDH activity, the

activity is low (He *et al.,* 2021). Genetic engineering techniques adopted for enhanced xylitol production in *S. cerevisiae* include (i) the disruption of impeding genes, (ii) the overexpression of endogenous genes, or (iii) the insertion of genes encoding key enzymes (Umai *et al.,* 2022).

1.4.2.2.1 Engineering strategies for the biotechnological production of xylitol by xylose reductase

Two common engineering strategies are applied in the creation of high-titre xylitol-producing *S. cerevisiae* namely, (i) the heterologous expression of the *S.s.xyl1* gene encoding XR from *Scheffersomyces stipitis* or (ii) the overexpression of the *S. cerevisiae GRE3* gene (de Mello *et al.,* 2022). The *GRE3* gene encodes a non-specific aldose reductase capable of reducing xylose to xylitol (Moysés *et al.,* 2016). It exhibits low enzyme activity in wild-type strains of *S. cerevisiae* (He *et al.,* 2021). However, when overexpressed GRE3 displays a higher enzyme activity than overexpressed *S.s.xyl1* in some strains (Konishi *et al.,* 2015).

Genes from *S. stipitis* have been extensively employed as a source for the oxidoreductase pathway for xylose consumption and occur in several of the best-performing yeasts engineered for xylose assimilation via this route (Lane *et al.,* 2018a). The XR from *S. stipitis* utilises both NADH and NADPH as cofactors leading to a cofactor imbalance between XR and XDH – particularly under anaerobic conditions since the oxidation of NADH cannot occur – which results in a surplus of NADH and consequently, an accumulation of xylitol (Cadete *et al.,* 2016; Kwak and Jin, 2017). Oh *et al.* (2013) reported that *S. cerevisiae* engineered to express *S.s.xyl1* can potentially generate xylitol at maximum theoretical yields (1.00 g xylitol per g xylose) due to the strain's inability to metabolise xylose, provided a co-substrate was supplied for metabolism and cell growth. To increase the production of XR, Tantirungkij *et al.* (1993) successfully subcloned *S.s.xyl1* into an expression vector with the constitutive enolase promoter ($ENO1_P$) and terminator ($ENO1_T$). The *S. cerevisiae* strain transformed with these plasmids demonstrated constitutive XR activity about 3 times greater than in *S. stipitis* and 20 times greater than that of the native promoter in *S. cerevisiae.* Most industrial xylitol production occurs from pure xylose substrate (Antunes *et al.,* 2022; Umai *et al.,* 2022). The different efforts for xylitol production by engineered *S. cerevisiae* is summarised in Table 1.3 below.

Table 1.3. Yields and titers of xylitol produced by engineered *S. cerevisiae* from lignocellulosic sugars. Adapted from (Lane *et al.,* 2018a).

Carbon sources	Highest yields	Highest titers (g/L)	References
Rice straw hydrolysate	0.79 g/g xylose	37.9	(Guirimand *et al.,* 2016)
Hemicellulosic hydrolysate of corncob	Near theoretical maximum	21	(Kogje and Ghosalkar, 2016)
Glucose and xylan	0.71 g/g xylan	1.94	(Li *et al.,* 2013b)
Cellobiose and xylose	1 g/g xylose	19.24	(Zha *et al.,* 2013)
Cellobiose and xylose	1 g/g xylose	93	(Oh *et al.,* 2013)
Glucose and xylose	0.96 g/g xylose	57	(Oh *et al.,* 2013)
Glucose and xylose	1 g/g xylose	196.2	(Jo *et al.,* 2015)
Glucose and xylose	1 g/g xylose	91.3	(Oh *et al.,* 2012)
Glycerol and xylose	1 g/g xylose	47	(Kogje and Ghosalkar, 2017)
Glucose and xylose	1 g/g xylose	21	(Lane *et al.,* 2018b)

1.4.2.2.2 Xylanolytic enzymes and engineering strategies for the bioproduction of xylitol from hemicellulose hydrolysates

The engineering of *S. cerevisiae* strains expressing xylanolytic enzymes is a promising strategy for efficient LCB utilisation since xylans are the dominant hemicellulose polymer in hardwoods and are the second most abundant polysaccharide found in nature (Choengpanya *et al.,* 2015; Katahira *et al.,* 2004; Procópio *et al*., 2022). Xylan is comprised of a backbone of repeating β-1,4-linked xylose units that are partially substituted with arabinosyl, acetyl, and glucuronosyl side chains (Katahira *et al.,* 2004; Procópio *et al*., 2022). Figure 1.7 displays the structure of xylan along with the xylan hydrolases required for its complete enzymatic degradation. As depicted in Figure 1.7, the enzymatic hydrolysis of xylan involves several hydrolytic enzymes attributable to its heterogeneous structure and high variability. These xylanolytic enzymes typically include acetylxylan-esterase, α-L-arabinofuranosidase, α-glucuronidase, β-

xylosidase, and endo-1,4-β-xylanase (Hilpmann *et al.,* 2019). Two important enzymes required to degrade the β-1,4-xylan backbone are endo-1,4-β-xylanases (EC 3.2.1.8), which hydrolyse xylan into short xylo-oligomers, and β-xylosidases (EC 3.2.1.37), which further degrade xylo-oligomers into xylose (Li *et al.,* 2013b; Van Zyl *et al.,* 2015). These enzymes are of further interest the research topic as pentose streams of pretreated LCB comprise a mixture of xylan, xylo-oligomers, and xylose (Lu *et al.,* 2021).

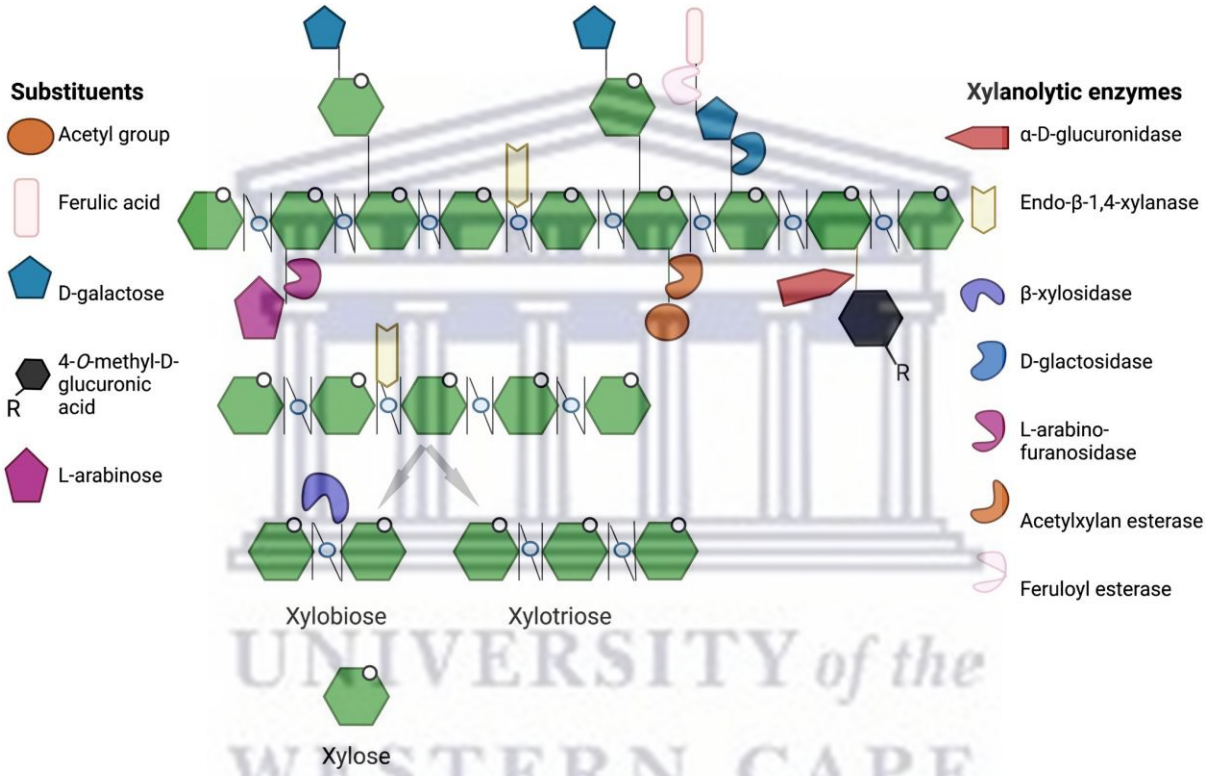

Figure 1.7. Schematic representation of the structure of xylan along with the enzymes required for xylan degradation (Kruger and Den Haan, 2022). The xylan backbone is initially hydrolysed by endo-1,4-xylanase into xylo-oligomers which are further degraded by β-xylosidase to produce xylose monomers.

On an industrial scale, the two above-mentioned hemicellulases are mainly produced by *Trichoderma* and *Aspergillus* fungi (Godoy *et al.,* 2018; Li *et al.,* 2013b; Zhu *et al.,* 2011). Fungi are beneficial for industrial xylanase production as their secreted enzyme levels are, usually, far greater than those of bacteria and yeast (Godoy *et al.,* 2018). *Trichoderma reesei* has been extensively exploited as a workhorse for xylanase production as it has been shown to express multiple xylanolytic enzymes belonging to different families and exhibiting distinct cleave specificity to xylan (Yan *et al.,* 2021). Of these xylanases, two main enzymes, accounting for

90 % of the secreted xylanase, are secreted when induced; namely endo-xylanase Xyn1 and Xyn2 belonging to the glycosyl hydrolase family 11 (GH11) (Herold *et al.,* 2013; Yan *et al.,* 2021). The GH11 endo-xylanase encoded by *xyn2* was reported to show higher stability and catalytic activity (Yan *et al.,* 2021).

Aspergillus niger is a well-researched filamentous fungus and owing to its high capacity for enzyme secretion and value for biotechnology, it serves as a good source of commercial enzymes including GH3 β-xylosidases (Choengpanya *et al.,* 2015; Sultan *et al.,* 2017). La Grange *et al.* (2000) co-expressed the *Bacillus pumilus* β-xylosidase (*B.p.xynB*) and *T. reesei T.r.xyn2* in *S. cerevisiae,* however, the engineered strain was unable to produce xylose from birchwood xylan. The authors presumed this to be due to the low XynB activity and the enzyme's low affinity for xylobiose. Consequently, the same group of researchers co-expressed the *A. niger* GH3 β-xylosidase (*A.n.xlnD*) with *T. reesei's T.r.xyn2* in *S. cerevisiae* which displayed synergistic hydrolysis of xylan (birchwood) to xylose with a 57 % conversion rate (La Grange *et al.,* 2001).

Mert *et al.* (2016) subsequently co-expressed *A.n.xlnD* and *T.r.xyn2* in a *S.cerevisiae* strain engineered for xylose consumption. Under limited oxygen conditions, the engineered strain produced ethanol at a maximum theoretical yield of approximately 90 % from xylose. Kruger and Den Haan (2022) used the same xylose-assimilating *S. cerevisiae* strain to co-express a *Pyrenophora tritici-repentis* β-xylosidase (*P.t.r.xln43*) with *T.r.xyn2*. The *P.t.r.xln43* encodes a GH43 β-xylosidase that was utilised to prevent the transglycosylation activity brought about by GH3 xylosidases. Additionally, the *P.t.r.xln43* displayed higher levels of enzyme activity in yeast than tested GH3 enzymes (Kruger and Den Haan, 2022). These results agree with Brevnova *et al.* (2011) who expressed the *P.t.r.xln43* in *S. cerevisiae* which yielded a xylosidase activity 6.9-fold higher than the *A. niger* GH3 *xlnD*, using the same expression vectors. The *S. cerevisiae* strain produced ethanol from xylan as its sole carbohydrate source (Brevnova *et al.,* 2011).

Guirimand *et al.* (2019) set out to engineer a xylitol-producing *S. cerevisiae* strain that displayed enhanced surface-tethered xylanase and xylosidase activity. This was achieved by means of a *SED1* gene cassette expressing the *SED1* promoter, *SED1* secretion signal, and

SED1 anchoring domain that led to an improved strain with a significantly increased xylitol production capacity compared to the previously engineered strain lacking the *SED1* cassette. Kruger and Den Haan (2022) utilized the *SED1* anchoring domain to express a surface-tethered xylosidase (*P.t.r.xln43-SED1*) and reported an increase in (i) enzyme production, (ii) growth capabilities on polymeric substrates and (iii) hemicellulosic conversion compared to strains with secreted xylosidase activity.

1.4.2.2.3 Engineering strategies for industrial yeast strains

Chromosomal integration and independently replicating plasmid vectors are broadly used to insert genes and regulate copy number in *S. cerevisiae* (Da Silva and Srikrishnan, 2012; Gnügge and Rudolf, 2017; Gu *et al.,* 2015). Since *S. cerevisiae* has very efficient homologous recombination repair systems, chromosomal integration of genes provides an alternate, direct technique for gene introduction as opposed to plasmids which offer restricted copy number control and have the possible issue of segregational instability. While plasmid vectors are best for the overexpression of genes, chromosomal integration is a key approach for metabolic engineering in yeast since it enables the (i) accurate control of expression, (ii) long-term stability, and (iii) insertion of multiple genes (Da Silva and Srikrishnan, 2012). Chen *et al.* (2018), integrated exogenous genes into the delta (δ) sequence of the genome of *S. cerevisiae* which resulted in an increase in the stability of target genes as well as their copy numbers. The authors suggested that this "δ-sequence-based integrative expression" approach be applied in the engineering of metabolic pathways in *S. cerevisiae*.

Delta sequences are long terminal repeats (LTRs) (300 bp) of the TY1 and TY2 retrotransposons in *S. cerevisiae*. Based on the genome of the *S. cerevisiae* S288c strain, 300 delta elements are distributed throughout the genome as single δ elements or linked to TY elements (Da Silva and Srikrishnan, 2012; Franco-Duarte e*t al.,* 2011). Therefore, δ integration provides a stable multi-copy integration into the chromosome throughout the genome, and in doing so, offers a strategy to overexpress genes of interest (Malci *et al.,* 2020). Qi *et al.* (2022) compared three multi-copy chromosomal integration strategies (episomal plasmids, δ- and rDNA integration) to produce the value-added product, caffeic acid. The strain constructed via δ integration displayed the highest caffeic acid production and led to a 50-

fold increase compared to the initial construct. They concluded that the delta-integrative technique is a promising approach to produce value-added bio-products in recombinant *S. cerevisiae*. Researchers aimed to further improve this method by coupling it with a CRISPR-mediated approach (Figure 1.8). Shi *et al.* (2016) efficiently integrated a large (24 kb) biochemical pathway in *S. cerevisiae* by coupling the δ-integration method with CRISPR-Cas which enabled a markerless integration and achieved a copy number 5.9-fold higher than that of traditional δ-integration. Huang and Geng (2020), similarly utilised a CRISPR-Cas9-mediated δ-integration strategy to engineer a 2,3-butanediol (2,3-BDO) biosynthesis pathway in *S. cerevisiae* and achieved a mean of 13.4 copies of the 2,3-BDO pathway compared to a mean of 7.5 copies when utilising traditional δ-integration. Moreover, the average titre of 2,3-BDO was almost 2-fold higher in CRISPR-mediated δ-integration strains than in conventional δ-integrated strains.

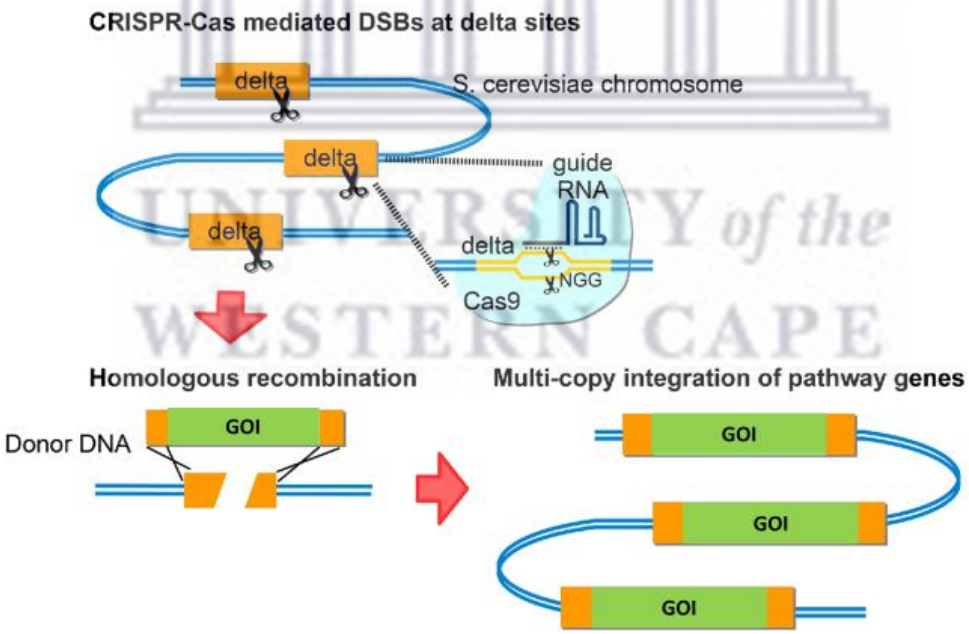

Figure 1.8. Schematic representation of CRISPR-Cas9-mediated δ-integration. Adapted from (Shi e*t al.*, 2019). CRISPR-Cas9 induces numerous double-stranded breaks (DSBs) in the δ sites throughout the *S.cerevisiae* genome. The gene of interest (GOI) is then inserted at the cleaved delta sites via the inherent homologous recombination mechanism of *S. cerevisiae*, thereby enabling multi-copy gene integration.

1.4.2.2.4 Ideal S. cerevisiae host strains for xylitol production from lignocellulosic hydrolysates

Industrially ideal CBP strains are required to have a range of traits including (i) satisfactory high levels of cellulase production, (ii) co-fermentation of pentoses and hexoses, (iii) tolerance to process changes, pretreatment-derived inhibitors and ethanol (or other products), and (iv) maximized productivity and product yield (Brethauer and Studer, 2015; Den Haan, 2018). However, no microorganism possessing this range of traits has been engineered or isolated (Den Haan, 2018). *S. cerevisiae* is deemed a prominent host for CBP considering its (i) genetic adaptability, (ii) high-cell-density fermentation capacities, (iii) rapid growth rate, (iv) GRAS status, (v) eukaryotic post-translational processing, and (vi) high tolerance to inhibitor compounds released in hemicellulosic hydrolysates (Davison *et al.,* 2019; Den Haan *et al.,* 2015; Den Haan, 2018; He *et al.,* 2021). However, domestic (industrial and laboratory) *S. cerevisiae* strains cannot secrete high titers of cellulolytic enzymes which represents a limiting point in CBP bioconversion technologies (Davison *et al.,* 2016; Davison *et al.,* 2019).

As a result, there is a developing interest in the exploitation of natural yeast isolates as opposed to laboratory yeast strains or strains employed in the production of 1G ethanol, since several of these isolates displayed more tolerance to inhibitors and other environmental stresses and could potentially have greater heterologous enzyme secretory capacity (Davison *et al.,* 2016; Jansen *et al.,* 2017). Examples of such natural isolates include the diploid, homothallic YI13 (high secretor), FIN1 (medium secretor), and YI59 (low secretor) *S. cerevisiae* strains obtained from coastal and inland winery regions of the Western Cape, South Africa, owing to the area's distinct environmental selection pressures (Davison *et al.,* 2016; Davison *et al.,* 2019). Strain selection is a crucial element to ensure the highest possible production of recombinant enzymes and as such, natural yeast isolates could offer a superior foundation for genetically engineering microbes needed for CBP and other industrial processes (Davison *et al.,* 2016; Den Haan, 2018).

1.5 RESEARCH OBJECTIVES

Xylitol, one of the top ten desired biochemicals with a well-established market, is an ideal biorefinery-based product since its conversion process is coupled to bioenergy and it is viable

for large-scale production (Bozell and Petersen, 2010; Budzianowski, 2017; Ravella *et al.,* 2022). The construction of a xylitol-producing *S. cerevisiae* strain represents an economically feasible and environmentally friendly approach to xylitol production (Mohite and Patil, 2016). Promising results have been achieved with *S. cerevisiae* engineered to produce xylitol, however insufficient studies focus on lignocellulosic hydrolysates as most industrial xylitol production occurs from pure xylose substrate (Antunes *et al.,* 2022; Baptista *et al.,* 2018; Umai *et al.,* 2022). Additionally, only a small number of strains have been considerably exploited as engineering hosts, despite the vast diversity of natural *S. cerevisiae* populations (Wang *et al.,* 2016). As a result, a *S. cerevisiae* strain is yet to be engineered with efficient substrate conversion properties and hydrolase-production capacity (Cunha *et al.,* 2020; Davison *et al.,* 2016; Den Haan, 2018).

Therefore, the aim of this research project was to engineer natural *S. cerevisiae* strains, previously shown to be resistant to pre-treatment-derived inhibitors, for the conversion of xylan, XOS, and xylose to xylitol. The synthesis of xylitol from hemicellulosic hydrolysate requires the co-ordinated and synergistic activity of 1,4-β-xylanase, 1,4-β-xylosidase, and xylose reductase, therefore the aim was realized via the following objectives:

- Individually integrated each enzyme-encoding gene (*S. stipitis xyl1*, *P. tritici-repentis xln43,* and *T. reesei xyn2*) into the δ-sequence of robust *S. cerevisiae* natural isolates (FIN1, YI13, and YI59). A CRISPR-Cas9-based method was used. The construction of single heterologous gene strains was done to test heterologous enzyme activities in the absence of other heterologous enzymes.
- Constructed three xylitol-producing strains (FIN1-X3, YI13-X3 and YI59-X3). A step-wise integration approach was adopted whereby *P.t.r.xln43* and *T.r.xyn2* were integrated via CRISPR-cas9-mediated transformation. *S.s.xyl1* was lastly integrated via conventional transformation.
- Tested the production of xylitol from xylose, xylan, xylo-oligosaccharides, and mixtures of xylan and xylo-oligosaccharides at shake flask level, in recombinant FIN1, YI13, and YI59 strains producing all three heterologous enzymes.
- Validated the common inhibitor tolerances of the naturally robust *S. cerevisiae s*trains.
- Analysed the metabolic burden exerted on recombinant FIN1, YI13, and YI59 strains.

CHAPTER 2

MATERIALS AND METHODS

All materials used in this study were distributed by Merck (Kenilworth, NJ, USA) or Sigma-Aldrich® (St. Louis, MO, USA), unless otherwise stipulated.

2.1 MICROBIAL STRAINS AND PLASMIDS USED IN THE STUDY

Competent *Escherichia coli* DH5α (Thermo Fisher Scientific) were used for subcloning. *E. coli* and yeast strains were stored in 40 % and 15 % (v/v) glycerol, respectively at – 80 °C. Tables 2.1 and 2.2 detail the yeast strains and plasmids used in this study, respectively.

Table 2.1. Details of yeast strains used in this study

MICROBIAL STRAIN	CONDENSED NAME	DESCRIPTION	REFERENCE
Scheffersomyces (Pichia) stipitis 5776	*S. stipitis* 5776	Type strain of native pentose-fermenting yeast	WH van Zyl laboratory, Stellenbosch University
S. cerevisiae FIN1	FIN1	Natural strain isolate	Davison *et al.,* 2016
S. cerevisiae YI13	YI13	Natural strain isolate	Davison *et al.,* 2016
S. cerevisiae YI59	YI59	Natural strain isolate	Davison *et al.,* 2016
S. cerevisiae FIN1 + pCas9-Nat	FIN1-Cas9	Natural yeast strain containing pCasNAT	Minnaar and Den Haan, 2023
S. cerevisiae YI13 + pCas9-Nat	YI13-Cas9	Natural yeast strain containing pCasNAT	Minnaar and Den Haan, 2023
S. cerevisiae YI59 + pCas9-Nat	YI59-Cas9	Natural yeast strain containing pCasNAT	Minnaar and Den Haan, 2023
S. cerevisiae FIN1 + pCas9-Nat + pRS42-G-DELTA + (*S.s.xyl1* + *P.t.r.xln43_SED1* + *T.r.xyn2*)	FIN1-X3	Xylitol-producing FIN1 with two xylanolytic enzymes (*P.t.r.xln43_SED1* and *T.r.xyn2*) and *S.s.xyl1* integrated in the delta sites of the genome	This study
S. cerevisiae YI13 + pCas9-Nat + pRS42-G-DELTA (*S.s.xyl1* + *P.t.r.xln43_SED1* + *T.r.xyn2*)	YI13-X3	Xylitol-producing YI13 with two xylanolytic enzymes (*P.t.r.xln43_SED1* and *T.r.xyn2*) and *S.s.xyl1* integrated in the delta sites of the genome	This study
S. cerevisiae Y159 + pCas9-Nat + pRS42-G-DELTA (*S.s.xyl1* + *P.t.r.xln43_SED1* + *T.r.xyn2*)	YI59-X3	Xylitol-producing YI59 with two xylanolytic enzymes (*P.t.r.xln43_SED1* and *T.r.xyn2*) and *S.s.xyl1* integrated in the delta sites of the genome	This study
S. cerevisiae YI13 + 11_SED1p_xyn2_DIT1t	YI13-xyn2	*S. cerevisiae* YI13 with *T.r.xyn2* integrated into	This laboratory

		chromosome 11; the gene is under the control of the *SED1* promoter and *DIT1* terminator	
S. cerevisiae Y159 + pCas9-Nat + pRS42-G-DELTA (*S.s.xyl1* + *P.t.r.xln43_SED1* + *T.r.xyn2*)	YI59-X(3-1)	Xylitol-producing YI59 with *T.r.xyn2* and *S.s.xyl1* integrated in the delta sites of the genome.	This laboratory

Table 2.2. Details of plasmids used in this study

PLASMID	BACTERIAL / YEAST MARKER	DESCRIPTION	REFERENCE
pBKD2	Ampicillin/G418	An expression vector for delta integration into *S. cerevisiae*; It carries the *ENO1* promoter and terminator	McBride *et al.,* 2008
pBKD2-XR	Ampicillin / G418	pBKD2 carrying *S.s.xyl1*	This study
pSED1p-DIT1t	Ampicillin / N/A	pUC57s plasmid carrying synthetic *SED1* promoter and *DIT1* terminator with *Pac*I-*Asc*I restriction sites in between to allow for cloning	Thermofisher Scientific
pRDH177_SED1	Ampicillin / ScURA3 and Zeocin	pMU1531 plasmid carrying the *ENO1* promoter and terminator, and *P.t.r.xln43_SED1*; Used to generate the *ENO1*p-*P.tr.xln43_SED1*-*ENO1*t cassette via PCR	Kruger and Den Haan, 2022
pRDH182	Ampicillin / ScURA3 and Zeocin	pMU1531 plasmid carrying the *ENO1* promoter and terminator, and *T.r.xyn2*; Used to generate the *ENO1*p-*T.r.xyn2*-*ENO1*t cassette via PCR	Brevnova *et al.,* 2011
pSED1-xln43_SED1-DIT1t	Ampicillin / N/A	pSED1p-DIT1t carrying the *Pac*I-*Asc*I, *P.t.r.xln43_SED1* cloned fragment	This study
pSED1-xyn2-DIT1t	Ampicillin / N/A	pSED1p-DIT1t carrying the *Pac*I-*Asc*I, *T.r.xyn2* cloned fragment	This study
pRS42-G-DELTA	Ampicillin / G418	Guide RNA scaffold plasmid targeting yeast DELTA sequences	Jacob *et al.,* 2022

2.2 YEAST AND PLASMID ISOLATION

All glycerol stock yeast strains (Table 2.1) were cultured on yeast peptone glucose (YPD) agar (1 % (w/v) yeast extract, 2 % peptone, 2 % glucose, and 2 % agar), supplemented with 100 µg/ml CloNAT (Jena Bioscience, Jena, Germany) and/or 200 µg/ml Geneticin (G418) sulphate (Invitrogen, Waltham, MA, USA) as required, for 2 days at 30℃. Resultant single colonies were inoculated into YPD media with the same prior supplementation and incubated overnight at 30 °C with an agitation of 180 rpm on an orbital shaker. All plasmids (Table 2.2) were propagated in *E. coli* DH5α and cultured on Luria-Bertani (LB) agar (0.5 % yeast extract, 1 % tryptone, 1 % sodium chloride, and 2 % agar), supplemented with 100 µg/ml ampicillin (amp) overnight at 37 °C. Resultant single colonies were inoculated into LB media supplemented with 100 µg/ml ampicillin and incubated overnight at 37 °C on a rotary wheel. Plasmid DNA was extracted from the overnight culture using the ZymoPURE™ Plasmid Miniprep kit (Zymo Research, Irvine, CA, USA) as directed by the manufacturer and quantified on a NanoDrop2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

2.3 PLASMID CONTRUCTION

Total DNA was extracted from *S. stipitis* 5776 overnight liquid cultures via Hoffman and Winston's (1987) DNA preparation method. The PCR analyses utilized Phusion DNA polymerase (Thermo Fisher Scientific) as directed by the manufacturer. Primers XYL1-L and XYL1-R (Table 2.3) were used for the amplification of *S.s.xyl1* in an Applied Biosystems thermocycler (Thermo Fisher Scientific). PCR reaction conditions were as follows: initial denaturation at 98 °C for 5 min, thereafter 31 cycles of denaturation at 98 °C for 30 s, annealing at 63.7 °C for 30 s, and elongation at 72 °C for 30 s; a final elongation at 72 °C for 7 min.

The miniprepped vector plasmids (pBKD2 and pSED1p-DIT1t) and plasmids housing genes of interest (GOIs) (pRDH177_SED1 and pRDH182) (Table 2.2), and the *S.s.xyl1* PCR product were individually subjected to *Pac*I-*Asc*I (Thermo Fisher Scientific) double digestion for 20 min at 37 °C, followed by incubation at room temperature for 1 hr. The digests were resolved by 1 % agarose gel electrophoresis (1 hr at 90 V) using a PowerPac™ Basic (Bio-Rad, Hercules, CA, USA) and viewed on a Dark Reader transilluminator (Claire Chemical Research, Dolores, CO, USA) where the appropriate plasmid vectors and GOIs were cut from the gel. The digested

DNA was then purified from the agarose gel using standard Phenol: Chloroform: Isoamyl alcohol (PCI) extraction. Purified vector plasmid and GOI DNA was ligated using T4 DNA ligase (Thermo Fisher Scientific) as directed by the manufacturer as follows: pBKD2 ligated with *S.s.xyl1*, pSED1p-DIT1t ligated with *P.tr.xln43_SED1 Pac*I-*Asc*I fragment, and pSED1p-DIT1t ligated with *T.r.xyn2 Pac*I-*Asc*I fragment. Samples were incubated at room temperature for 1 hr, followed by 30 min at 4 °C. The ligated DNA was dialysed for 5 min on an MF-Millipore™ 0.025 µm MCE Membrane against demineralised distilled water.

Competent *E. coli* DH5α was subjected to transformation with the dialysed DNA via electroporation to propagate the cloned plasmids: *E. coli* DH5α was combined with dialysed DNA in an ice-cold electroporation cuvette (Bio-Rad) and transformed using a MicroPulser (Bio-Rad) (2.5 kV, 25 µF capacitance, 200 ohm resistance). Thereafter, cells were resuspended in super optimal broth with catabolite repression (SOC) media (0.5 % yeast extract, 2 % tryptone, 0.058 % sodium chloride, 0.019 % potassium chloride) and incubated for 1 hr at 37 °C, with agitation. The transformation mixture was plated on LB agar supplemented with ampicillin (100 µg/ml) and incubated overnight at 37 °C. Successful transformants were confirmed by isolating plasmid DNA from the resultant colonies on the LB-amp agar according to the preceding protocol outlined in subsection 2.2. Isolated plasmid DNA was *Pac*I-*Asc*I double digested and resolved by gel electrophoresis under the same conditions previously described. The 1 % agarose gel was viewed using an Enduro™ GDS system (Labnet, Edison, NJ, USA) to identify successful cloned plasmids: *P.tr.xln43_SED1* and *T.r.xyn2* cloned to separate pSED1p-DIT1t plasmids and *S.s.xyl1* cloned to pBKD2 (see Table 2.2).

Table 2.3. Details of primers used in this study

PRIMER	SEQUENCE (5' TO 3' DIRECTION)	RESTRICTION SITE	APPLICATION
DELTA-ENO1-L	CTTAAGATGCTCTTCTTATTCTATTAAAAATAG AAAATGACTTCTAGGCGGGTTATCTACTG	NONE	Amplification of genes under the control of the *ENO1* promoter and terminator for delta integration
DELTA-ENO1-R	GTTTGTTTGCGAAACCCTATGCTCTGTTGTTCG GATTTGACGTCGAACAACGTTCTATTAGG		
ENO1-L	GTAACATCTCTCTTGTAATCCCTTATTCCTTCTA GC	NONE	Confirmation of genes in between *ENO1* promoter and terminator sequences in transformants
ENO1-R	GCAACCCTATATAGAATCATAAAACATTCGTG A		
XYL1-L	GTACTTAATTAAATGCCTTCTATTAAGTTGAAC TCTGG	*Pac*I	Amplification of *S.s.xyl1* from *S. stipitis* gDNA; enables *Pac*I-*Asc*I subcloning; Use ENO1-L with XYL1-R to confirm the presence of the *ENO1*p-*S.s.xyl1*-*ENO1*t cassette in transformants
XYL1-R	TGACGGCGCGCCTTAGACGAAGATAGGAATC TTGTCCC	*Asc*I	
DELTA_SED1p-L	CTTAAGATGCTCTTCTTATTCTATTAAAAATAG AAAATGAATTGGATATAGAAAATTAACGTAAG GCAGTATC	NONE	Amplification of genes under the control of the *SED1* promoter and *DIT1* terminator for delta integration
DELTA_DIT1t-R	GTTTGTTTGCGAAACCCTATGCTCTGTTGTTCG GATTTGATTACTCCGCAACGCTTTTCTG		

2.4 PCR AMPLIFICATION OF THE GENE CASSETTES

All PCR reactions utilised Taq DNA Polymerase Master Mix RED (Ampliqon, Odense, Denmark) as directed by the manufacturer. Plasmids pBKD2-XR, pSED1p-xln43_SED1-DIT1t, and pSED1p-xyn2-DIT1t (Table 2.2) were used to amplify the gene cassettes carrying *Scheffersomyces stipitis xyl1* (*S.s.xyl1*), *Pyrenophora tritici-repentis xln43* with the *SED1* anchoring domain (*P.tr.xln43_SED1*), and *Trichoderma reesei xyn2* (*T.r.xyn2*), respectively. Table 2.3 details the specific primers that were utilised to achieve the amplifications in an Applied Biosystems thermocycler. PCR reaction conditions for the amplification of the pBKD2-XR gene cassette were as follows: initial denaturation at 95 °C for 5 min, thereafter 31 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 1 min; a final elongation at 72 °C for 7 min. PCR reaction conditions for the amplification of the pSED1p-xln43_SED1-DIT1t and pSED1p-xyn2-DIT1t gene cassettes were as follows: initial denaturation at 95 °C for 2 min, thereafter 31 cycles of denaturation at 95 °C for 30 s,

annealing at 60 °C for 30 s, and elongation at 72 °C for 3 min and 20 s; a final elongation at 72 °C for 7 min. Amplification was confirmed by resolving PCR products on 1 % agarose gel (90 V for 1 hr) before products were purified using standard PCI extraction. This was followed by quantification on a NanoDrop2000 Spectrophotometer.

2.5 YEAST TRANSFORMATION

All host *S. cerevisiae* cells were transformed via the electroporation method described by Cho *et al.,* (1999), with slight modifications to improve the electrocompetence of cells (Moriguchi *et al.,* 2016). In brief, yeast cells were made competent by washing the harvested overnight culture with demineralised distilled water, prior to resuspension in 800 µL LiOAc/TE solution (0.1 M LiOAc, 10 mM Tris-HCl pH 8, 1 mM EDTA). The suspension was incubated at 30 °C for 45 min with gentle shaking, followed by the addition of 20 µL 1 M DTT (Millipore) and incubation at the same temperature for 15 min. Cells were subsequently harvested from the mixture and first washed with demineralised deionised water then with electroporation buffer (1 M sorbitol, 20 mM HEPES). Washed cells were resuspended in electroporation buffer. All plasmid and repair template DNA were dialysed according to the same protocol described in section 2.3, prior to electrotransformation. Competent cells were transformed with *Bst*1107I (Thermo Fisher Scientific) linearised pBKD2-XR (conventional transformation) or 5 – 10 µg repair template DNA and 1 µg CRISPR plasmid DNA (pRS42-G-DELTA) (CRISPR-Cas9-based transformation) using a MicroPulser (1.4 kV, 25 µF capacitance, 200 ohm resistance). Thereafter, cells were resuspended in YPD media supplemented with 1 M sorbitol, followed by 3 hr incubation at 30 ̊C with agitation 180 rpm. The transformation mixture was plated (100 µL) on YPD agar supplemented with either 100 µg/ml CloNAT and 200 µg/ml Geneticin (G418) sulphate or 200 µg/ml G418 only as required and incubated for 2 days at 30 °C. The remaining transformation mixture was left to incubate overnight at 30°C with agitation at 180 rpm before it was plated and allowed to cultivate in the same way.

2.5.1 Single Gene Transformation

The delta vector (pBKD2-XR) was linearised with *Bst1107I* for 20 min at 37 °C, followed by incubation at room temperature for 1 hr. The linearised plasmid DNA was purified using standard PCI extraction and then used to conventionally transform FIN1, YI13 and YI59.

Selection was achieved using YPD agar supplemented with 200 µg/ml G418, followed by sub-cultivation under the same selection. Putative positive transformants were inoculated in YPD media supplemented with 200 µg/ml G418 for pre-screening.

Separate yeast strains (FIN1-Cas9, YI13-Cas9, YI59-Cas9) were transformed with one gene cassette (*S.s.xyl1*, *P.t.r.xln43_SED1* or *T.r.xyn2*) and pRS42-G-DELTA (for delta integration) to confirm the respective enzyme activities in the absence of other recombinant enzymes. Transformants were selected on YPD agar supplemented with 100 µg/ml CloNAT and 200 µg/ml G418, followed by sub-cultivation under the same selection. Putative positive transformants were inoculated in YPD media supplemented with 100 µg/ml CloNAT and 200 µg/ml G418 for pre-screening.

2.5.2 Construction of Xylitol-Producing *S. cerevisiae*

Three diploid yeast strains (FIN1-Cas9, YI13-Cas9, YI59-Cas9) were transformed according to the preceding protocol, but with the intention of introducing all three genes (*S.s.xyl1*, *P.t.r.xln43_SED1*, *T.r.xyn2*) into the yeast's delta sequences, in consecutive rounds of transformation. Strain construction began by transforming all three yeast strains with *P.t.r.xln43_SED1* and pRS42-G-DELTA. Gene integration was confirmed with PCR and enzymatic assay pre-screening. Positive transformants were sub-cultured on YPD agar supplemented with 100 µg/ml CloNAT only to maintain the Cas9 plasmid but cure the G418 selective pRS42-G-DELTA. Plates were incubated for 2 days at 30 °C. Transformants were sub-cultured in five successive rounds. Following the fifth sub-cultivation, single colonies from the original transformation plates and the fifth sub-cultured plates were streaked onto YPD agar supplemented with 200 µg/ml G418 to confirm the absence of pRS42-G-DELTA in the final sub-cultured strains. Sub-cultured strains with recombinant *P.t.r.xln43_SED1* and cured pRS42-G-DELTA were transformed with *T.r.xyn2* and pRS42-G-DELTA using the same protocol as previously described. Following confirmation of gene integration via PCR and enzymatic assay pre-screening, positive transformants were sub-cultured as previously described. Sub-cultured strains cured of pRS42-G-DELTA and housing recombinant *P.t.r.xln43_SED1* and *T.r.xyn2* underwent a final transformation with *S.s.xyl1* according to the conventional transformation methodology previously explained. Transformation was confirmed with PCR and enzymatic assay pre-screening. Preliminary enzymatic assays conducted after each round

of transformation were also used to identify the strains with the highest enzyme activity profiles.

2.6 CONFIRMATION OF GENE-INTEGRATION AND ENZYMATIC ASSAYS

Transformants were screened by amplifying the respective gene cassettes via colony PCR. PCR reaction conditions for the amplification of the *SED1*p-*xln43_SED1-DIT1*t and *SED1*p-*xyn2-DIT1*t gene cassettes were repeated as previously described in section 2.4. The ENO1-L and XYL1-R primers (Table 2.3) were used to amplify the *ENO1*p-*S.s.xyl1-DIT1*t gene cassette under the same PCR conditions applied to amplify the cassette from pBKD2-XR as previously described in section 2.4. PCR products were resolved by gel electrophoresis as previously described and viewed using an Enduro^{TM} GDS system to determine the presence or absence of gene cassettes in the yeast cells.

Xylose reductase activity was preliminary screened following adapted methodologies (Yokoyama *et al*., 1995; Eliasson *et al.,* 2000; Çağlayan and Wilson, 2014). Single colonies of putative transformed cells were inoculated into 5 mL YPD media overnight at 30 °C with agitation 180 rpm. Thereafter, cells were sub-cultured into fresh YPD media in a 1:10 dilution and incubated under the same conditions until yeast cells reached log phase. Cells were then harvested and washed with demineralised distilled water prior to being washed with 25 mM potassium phosphate buffer (pH 8). Washed cells were resuspended in lysis buffer (25 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM NaCl, 10 mM β-mercaptoethanol) and 0.5 mm diameter acid-washed glass beads were added in a 1:1 ratio. The glass bead suspension was vortexed as follows: 6 cycles of 30 s vortexed at maximum speed with alternating cycles of 1 min cooling on ice. To remove cell debris, the vortexed mixture was centrifuged at full speed for 20 min at 4 °C and the supernatant (yeast cell extract) was used for subsequent XR activity analysis. The reaction mixture was set-up on ice as follows: 150 mM potassium phosphate buffer, 0.2 mM NADPH, 350 mM xylose and 10 or 20 µL yeast cell extract made up to a final volume of 200 µL with dH_2O. The reaction mixture was set-up excluding the yeast cell extract and all reagents were equilibrated to 30 °C while the yeast cell extract incubated at 4°C. A positive control was set-up which utilised *S. stipitis* 5776 cell extract. A negative control was set-up which excluded 350 mM xylose. The addition of the yeast cell extract to the reaction mixture began the assay and absorbances were measured every 30 s for 15 min at 340 nm using a

FLUOstar Omega Microplate Reader (BMG LABTECH, Ortenberg, Germany). The change in absorbance and Beers Law was used to measure the oxidation of NADPH which indirectly measured XR activity and confirmed the presence of *S.s.xyl1* (see Appendix 2).

Xylose reductase activity was determined by measuring the xylitol production of strains grown in YP media supplemented with glucose and 2 % xylose incubated at 30 °C for 72 hours. Strains were cultivated in a 1:1 and 1:10 glucose to xylose ratio, maintaining 2 % xylose and altering the glucose levels accordingly. Xylitol production was measured using a D-Sorbitol/Xylitol Assay Kit (Megazyme) as directed by the manufacturer's microplate assay procedure. D-Sorbitol standard curves were set with concentrations ranging from 0.48 to 14.3 mg/L (Appendix 1, Figure S3).

Preliminary enzymatic assays were performed where single colonies of putative positive transformants were inoculated in 5 ml YP media supplemented with 2 % glucose and cultivated for 72 hours at 30 °C at 180 rpm, alternatively enzyme assays occurred in final volumes of 10 mL YPD media with the same conditions. To confirm xylosidase activity, assays were performed using *p*-nitrophenyl-β-D-xylopyranoside (pNP-X) as a substrate as previously described by Kruger and Den Haan, 2022. The reaction mixture was set-up using total cell culture, 250 mM pNP-X and 50 mM NaOAc buffer (pH 5) in a 5:1:44 ratio made up to a final volume of 100 µL. Reactions occurred at 50 °C for 30 min and were stopped by the addition of an equal volume of 1 M Na_2CO_3. A yellow colour change confirmed positive *xln43_SED1* transformants. For quantification of activity, once the reactions were terminated by the addition of 1 M Na_2CO_3, the cultures were centrifuged, and the absorbance of assay supernatant was measured at 400 nm by a FLUOstar Omega Microplate Reader. The data was compared to a *p*-nitrophenyl (pNP) standard curve set between 0.075 and 1.25 mM pNP to determine the liberated amount of pNP as a measure of xylosidase activity (Appendix 1, Figure S1).

Confirmation of xylanase activity was established through a qualitative analysis whereby single colonies of putative positive transformed cells were spot plated onto SC^{-HIS} agar supplemented with 0.1 % Azurine cross-linked (AZCL) -xylan (Megazyme, Bray, Ireland). Plates were incubated overnight at 30 °C. Background yeast strains (FIN1-Cas9, YI13-Cas9, YI59-Cas9) served as negative controls for the respective transformants while YI13-xyn2 served as

a positive control. Colonies exhibiting xylanase activity were surrounded by a dark blue halo. To quantify xylanase activity, assays were performed according to the dinitrosalicylate (DNS) protocol (Bailey *et al.* 1992) using 1 % beechwood xylan (Megazyme) as a substrate. The culture supernatant was incubated with the xylan substrate for 5 min at 50 °C, after which DNS was added and incubation continued for 5 min at 90 °C then 1 min at 4 °C. The supernatant, xylan and DNS was combined in a ratio of 1:5:8 to a final volume of 140 µL. Background sugars were ascertained by adding DNS to the supernatant before the substrate was added to the reaction mixture. This was followed by incubation for 5 min at 90°C then 1 min at 4 °C. The absorbance of each reaction mixture was measured at 540 nm using a FLUOstar Omega Microplate Reader. A xylose standard curve was established using concentrations set between 0.5 and 10 g/L (Appendix 1, Figure S2) to determine the amount of liberated xylose as a measure of xylanase activity.

Following incubation of cultures for the quantified xylosidase and xylanase assays, the OD_{600} readings were recorded for all strains involved and was used to determine the dry cell weight (DCW) of yeast strains (Meinander *et al.,* 1996). The heterologous xylanase and xylosidase activities, were quantified as units per gram DCW (U/gDCW), where one unit was defined as the amount of enzyme that will produce reducing sugars (for xylanase) or p-nitrophenol (for xylosidase) at a rate of 1 µmol/minute under the assay conditions (Ghose and Bisaria, 1987). These values were standardised using the DCW of each strain. All assays were carried out in biological and technical triplicates and results are provided as averages of these triplicates with standard deviation specified.

2.7 ASSAYING XYLITOL PRODUCTION FROM DIFFERENT C5-SUGAR SOURCES

The xylitol production of constructed xylitol-producing strains (FIN1-X3, YI13-X3, YI59-X3; Table 2.1) was determined using the D-Sorbitol/Xylitol Assay Kit (Megazyme) as described above in the xylose reductase activity determination methodology. Xylitol production was measured from total cell cultures grown in YP supplemented with either 2 % xylan, 2 % XOS, a combination of 2 % xylan and 2 % XOS, or 2 % xylose incubated at 30 °C for varying timepoints ranging from 24 hours to 96 hours. Strains were cultivated in a 1:1 and 1:10 glucose to C5-sugar ratio, maintaining 2 % C5-sugar and altering the glucose levels accordingly. Glucose was used as a co-substrate. All xylitol assays were carried out in

biological triplicates with technical samples and results are presented as averages of these triplicates with standard deviation specified.

2.8 NATURAL TOLERANCE VALIDATION

Stress plate assays were performed to confirm the robustness of our strains to varying temperatures (30 °C, 37 °C, 40 °C) and acetic acid concentrations (3 g/L, 5 g/L). Single colonies of engineered xylitol-producing strains (FIN1-X3, YI13-X3, YI59-X3) and background strains (FIN1-Cas9, YI13-Cas9, YI59-Cas9) were inoculated into 10 mL YPD media and incubated at 30 °C for 2 days with an agitation of 180 rpm. Subsequently, cultures were standardised to an OD_{600nm} of 1 to a final volume of 1 mL with YPD media. Six 10-fold serial dilutions were then performed, using demineralised distilled water and 3 µL of each dilution was spotted onto YPD agar with the appropriate inhibitor. All spotted agar plates were incubated for 2 days; the 37 °C and 40 °C plates were incubated accordingly, and the remaining plates were incubated at 30 °C.

2.9 GROWTH CURVE ANALYSES

The growth and proliferation of the engineered and background isolates were monitored according to the protocol described by Chetty *et al.,* (2022). In brief, single cells were inoculated into YPD media and incubated overnight at 30 °C with an agitation of 180 rpm on an orbital shaker. The overnight cultures were inoculated to OD_{600nm} = 0.0567 in 10 mL YPD flasks and incubation continued under the same conditions. OD_{600nm} readings were recorded every 2 hours until stationary phase was reached, using a FLUOstar Omega Microplate Reader (BMG LABTECH, Ortenberg, Germany). Suitable dilutions were made for each OD_{600nm} reading. Growth curve analyses occurred in biological and technical triplicates and the OD_{600nm} values were presented as averages of the triplicates with error bars representing standard deviations.

2.10 STATISTICAL ANALYSES

Significant differences between quantitative data attained were determined using the two-tailed t-test, assuming unequal variance, where $p \leq 0.05$ was considered significant.

CHAPTER 3

RESULTS AND DISCUSSION

This study aimed to engineer a xylitol production route in natural *S. cerevisiae* strains in an effort to allow the manufacture of a high value co-product in a biorefinery. Three exogenous genes namely, *P.tr.xln43_SED1*, *T.r.xyn2* and *S.s.xyl1*, encoding a β-xylosidase, β-xylanase and a xylose reductase, respectively, were successively incorporated into the delta sequences of the natural host strains and were constitutively expressed to convert different C5-sugar sources into xylitol.

3.1 STRAIN CONSTRUCTION

The sustainability and viability of the cellulosic ethanol industry is dependent on adding value to its process residues (Tana *et al.,* 2021). Progress in metabolic and genetic engineering have driven the development of microbial cell factories (MCF) able to utilise lignocellulosic feedstock to produce value-added products, such as xylitol (Singhania *et al.,* 2022). An efficient xylitol-producing strain metabolises xylose into xylitol from hemicellulose hydrolysate using key enzymes that cooperatively interact in the metabolic route namely, xylose reductase (for the reduction of xylose to xylitol) and xylanolytic enzymes, for the hydrolysis of xylan to xylose (Katahira *et al.,* 2004; Manjarrés-Pinzón, 2022). *S. cerevisiae* strains are often bioengineered to produce lignocellulolytic activity and have been extensively researched for its promise for CBP. It is an excellent host for hydrolytic enzyme production and for its fermentative ability (Singhania *et al.,* 2022). As such, we transformed three natural isolate diploid *S. cerevisiae* strains reported to display high (YI13), medium (FIN1) and low (YI59) heterologous enzyme secretory phenotypes as well as varying degrees of robustness toward fermentation inhibitors (Davison *et al*., 2016; Davison *et al*., 2019).

We began with the construction of pBKD2-XR whereby *S.s.xyl1* was cloned between the $ENO1_P$ and $ENO1_T$ of pBKD2 – an *S. cerevisiae* delta integration expression vector placing *S.s.xyl1* under transcriptional control of the native glycolytic *ENO1* promoter and terminator. Construction of the pBKD2-XR plasmid was successful, as is evident from Figure 3.1. Both the pBKD2 vector of 5994 bp and the *S.s.xyl1* gene of 958 bp (lane 7) corresponds to the expected

size on the molecular weight marker (lane 1) as well as the linearised plasmid (lane 3) and *S.s.xyl1* PCR product (lane 5).

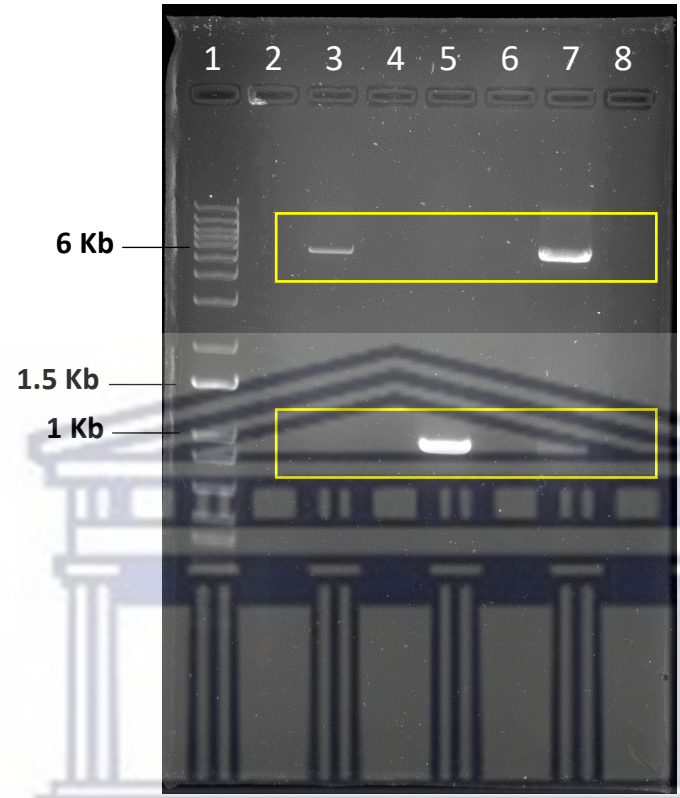

Figure 3.1. 1 % agarose gel depicting the constructed pBKD2-X2 recombinant vector. Lane 1: 1 Kb plus DNA ladder (Thermo Fisher Scientific); Lane 3: linearised pBKD2; Lane 5: *S.s.xyl1* PCR product; Lane 7: pBKD2-XR double digested with *Pac*I and *Asc*I.

To identify the better approach for the integration of the xylose reductase gene, we utilised both conventional (plasmid based) and CRISPR-Cas9-based transformation strategies on one of our natural isolates. *S.s.xyl1* was introduced into the YI59 host strain via multicopy δ-integration. Pre-screening xylose reductase assays were performed and the comparison of the two transformation strategies revealed that conventionally transformed strains exhibited greater xylose reductase activity (Appendix, Table S1). Thereafter, strain construction began by first creating single gene expression strains and confirming activity via pre-screenings, before manufacturing the strains containing all three genes.

Strain construction began by introducing the cell-tethered β-xylosidase gene, *P.t.r.xln43_SED1*, and secreted endo-β-xylanase gene, *T.r.xyn2*, into the δ regions of all three host strains' chromosomes, in consecutive rounds of transformation. A two-plasmid CRISPR-Cas9 system

was used whereby the Cas9 and gRNA were expressed from different vectors (Jacob *et al.,* 2022; Zhang *et al.,* 2014). Our host strains were previously transformed with the plasmid pCas9-NAT to yield FIN1-Cas9, YI13-Cas9 and YI59-Cas9 (Minnaar and Den Haan, 2023). This two-plasmid system resulted in an almost 100 % positive rate of directed genome editing in a haploid *S. cerevisiae* strain (DiCarlo *et al.,* 2013). Following confirmation of heterologous gene integration as detailed below, the gRNA plasmid was cured from each strain with five successive rounds of subculturing on YPD media supplemented with CloNAT, before the next round of transformation began. Lastly, the plasmid pBKD2-XR was then conventionally transformed into the recombinant strains via multicopy δ-integration for the incorporation of the xylose reductase encoding gene, *S.s.xyl1*, creating the FIN1-X3, YI13-X3 and YI59-X3 strains discussed below.

3.2 STRAIN CONFIRMATION

To confirm the success of the strain construction, presumptive positive transformants were subjected to qualitative analyses via both PCR confirmation, where the products were viewed on 1 % agarose gels and an AZCL-xylan plate assay. The successful integration of the three genes was confirmed via PCR analyses while the AZCL-xylan plate assay was used to confirm the successful integration of *T.r.xyn2*.

The relevant primers described in Table 2.3 were used to amplify the GOIs within the engineered strains. Confirmation of the xylose reductase gene (*S.s.xyl1*) was indicative of a 958 bp band in Figure 3.2a. The presence of *T.r.xyn2* and *P.t.r.xln43_SED1* were similarly confirmed (not shown). AZCL-xylan is produced by cross-linking xylan polysaccharides to render them insoluble, after which the polysaccharides are dyed. Upon cleavage by a xylanase, dyed xylo-oligosaccharide products diffuse into the agar, indicated by the formation of dark blue halos (Kračun *et al.,* 2015; Yan *et al.,* 2021). Our putative positive transformants (FIN1-X3, YI13-X3, YI59-X3) were spotted alongside their respective host strains (FIN1-Cas9, YI13-Cas9, YI59-Cas9) and a strain with proven xylanase activity (YI13-xyn2) was used as a positive control. The production of xylanase yielded dark blue halos, which were evident around the positive control strain and all three of our transformants (Figure 3.2b). The lack of dark blue zones surrounding the background strains exhibited their lack of xylanase activity.

The agarose gel images, and AZCL-xylan plate revealed that the three heterologous enzyme integrations were successful. Positive colonies correlating to both the agarose gel image and AZCL-xylan plate were then cultivated and their activities evaluated at shake flask level.

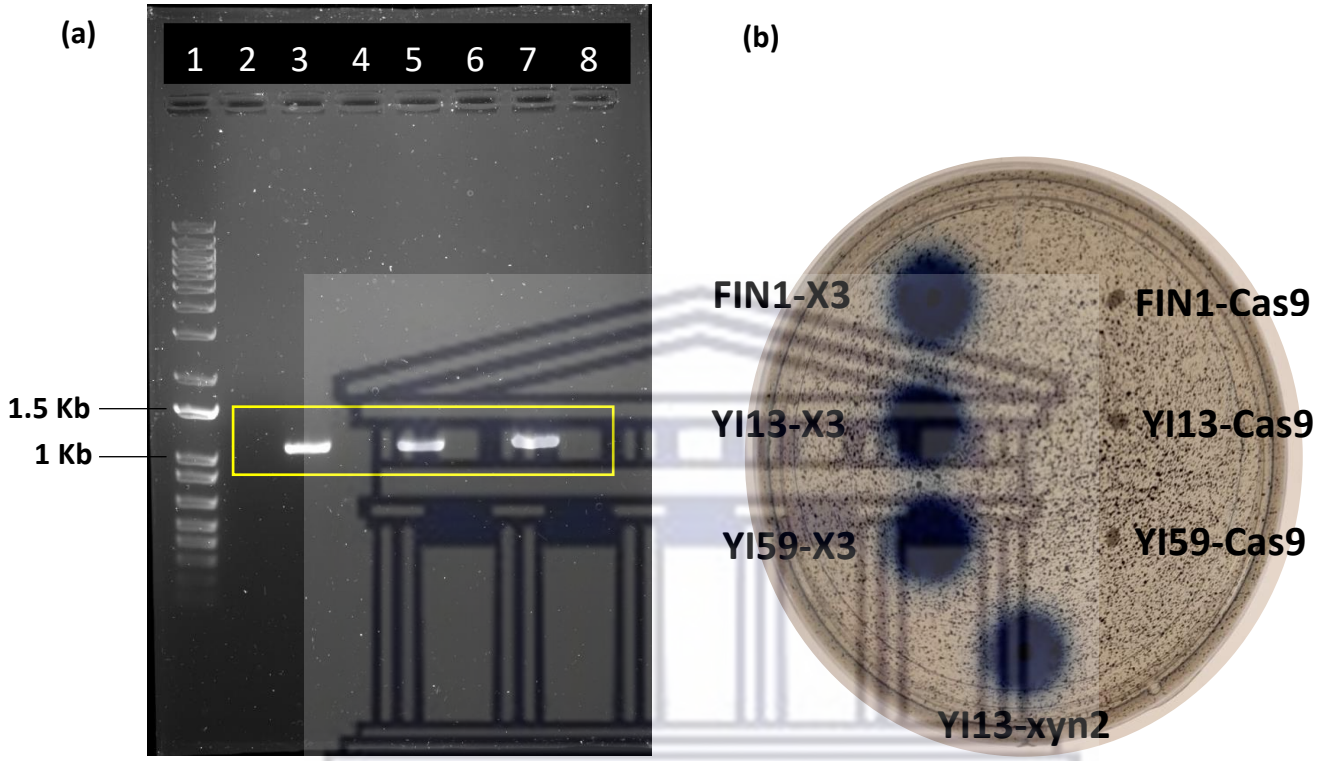

Figure 3.2. Qualitative analyses of heterologous gene integration. (a) 1 % agarose gel image illustrating the XR gene integration in the engineered strain isolates (FIN1-X3, YI13-X3, YI59-X3). Lane 1: 1 Kb plus DNA ladder; Lane 2: PCR negative control; Lane 3: FIN1-X3 *S.sxyl1*; Lane 4: FIN1-Cas9; Lane 5: YI13-X3 *S.sxyl1*; Lane 6: YI13-Cas9; Lane 7: YI59-X3 *S.sxyl1*; Lane 8: YI59-Cas9. (b) AZCL-xylan plate assay demonstrating the xylanase activity of our engineered (-X3) strains. Xylanase activity is presented as dark blue halos in all engineered strains (FIN1-X3, YI13-X3 and YI59-X3). YI13-xyn2 was included as a positive control.

3.3 ANALYSIS OF XYLOSE REDUCTASE ACTIVTY

The industrially important XR enzyme catalyses the reduction of xylose to xylitol (Lugani and Sooch, 2020). Accordingly, we set out to quantify the *S. stipitis* xylose reductase ability conferred to our strains. Xylose reductase activity was indirectly determined by quantifying the xylitol produced from 2 % xylose. In our engineered *S. cerevisiae* strains, the entire flux of xylose is directed towards xylitol generation, which cannot be consumed for cell growth and maintenance. Consequently, an additional substrate is necessary to maintain cell growth and metabolism (Lane e*t al.,* 2018a). Many researchers have used glucose as a co-substrate for

xylitol production (Jo *et al.,* 2015; Lane *et al.,* 2018b; Li *et al.,* 2013b; Oh *et al.,* 2012; Oh *et al.,* 2013). Similarly, we cultured our strains using glucose as a co-substrate. Since cultivation conditions is one of the factors affecting the yield of heterologous proteins (Cho *et al.,* 2022), we set out to determine the xylitol production of our engineered strains at three time points and under different glucose concentrations to determine when production was at its best. Xylitol titres were assayed after 72 hours (Figure 3.3.1), and a separate cultivation was examined after 24 and 96 hours (Figure 3.3.2). We compared the amount of xylitol produced by strains cultivated in a 1:1 and 1:10 glucose to xylose ratio, maintaining 2 % xylose and altering the glucose levels accordingly. Figure 3.3.1 and 3.3.2 illustrates and compares the xylitol titres produced by our engineered and background strains. Additionally, the rates of conversion to xylitol were calculated and presented in Table 3.1.

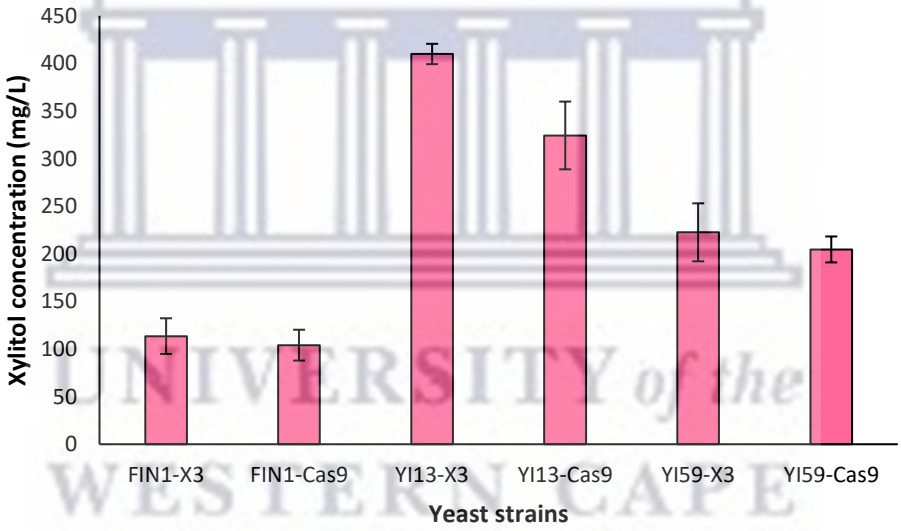

Figure 3.3.1. Xylitol production of metabolically engineered (-X3) and background (-Cas9) yeast strains after 72h cultivation on 2 % xylose at 30 °C. The media was supplemented with 2 % glucose as a co-substrate. Xylitol concentrations are expressed as mg/L and are shown as averages of the assays performed in triplicate with error bars representing standard deviations.

From Figure 3.3.1 it is immediately apparent that the background strains produced xylitol titres similar to their respective engineered strains. The unpaired t-test assuming unequal variance confirmed this observation and revealed that all background strains did not produce xylitol titres that were statistically different from their respective engineered strains. It

appears as though the overexpressed *S.s.xyl1* did not improve on the xylose reduction ability of the strains, likely conferred by the native *GRE3* gene.

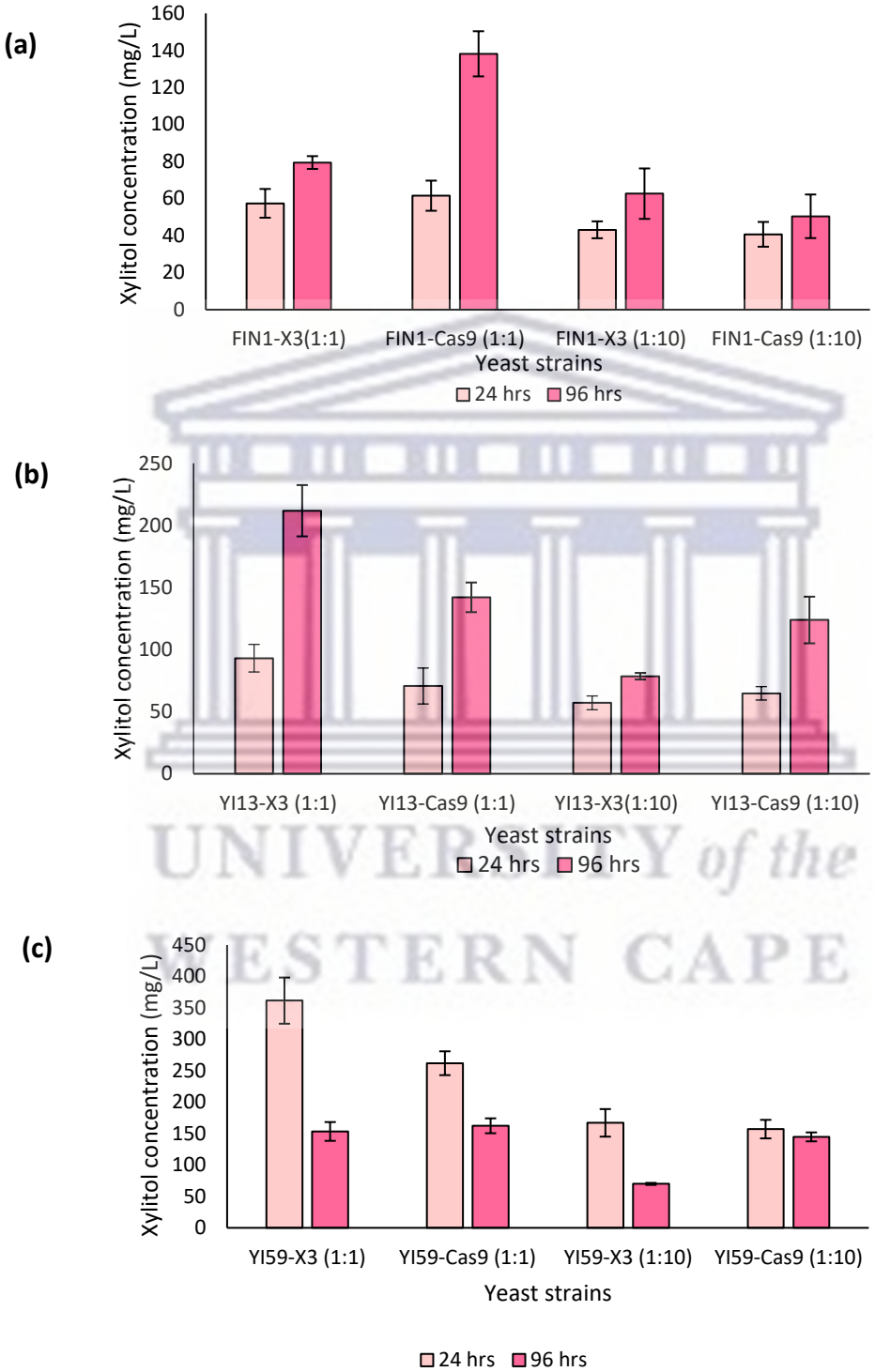

Figure 3.3.2. Xylitol production of metabolically engineered (-X3) and background (-Cas9) yeast strains after 24 and 96 h cultivations on 2 % xylose at 30 °C. Graphs (a), (b) and (c) illustrate the xylitol titres produced by FIN1, YI13 and YI59 strain isolates, respectively. The media was supplemented with either 2 % (1:1) or 0.2 % (1:10) glucose as a co-substrate. Xylitol concentrations are expressed as mg/L and are shown as averages of the enzyme assays performed in triplicate with error bars representing standard deviations.

As with Figure 3.3.1, comparison of the xylitol titres between the engineered and respective background strains in Figure 3.3.2 appeared indistinguishable. The unpaired t-test revealed this to be true for most strains. However, after 96 hours, FIN1-X3 (1:1), YI13-X3 (1:1), and YI59-X3 (1:10) produced xylitol titres statistically different from their respective background strains. The differeing values between Figures 3.3.1 and 3.3.2 are likely due to the batch variation between the kits used, however, overall analyses of both Figures led us to conclude that cultivation after 72 and 96 hours appeared to be suitable timepoints for assaying xylitol production. Analyses of the background strains in both Figures 3.3.1 and 3.3.2 revealed the native xylose reductase activity was unique to each strain, and comparison of the engineered and respective background strains disclosed that, for the most part, our engineering strategies were limited by the specific *GRE3* activity of the host strains.

He e*t al.* (2021) integrated *S.s.xyl1* into delta sequences of *S. cerevisiae* strains to enhance its native xylitol producing capacity since *GRE3* activity is usually low in wild-type strains. The natural strain isolates used in this study, however, displayed higher than expected native aldose reductase activity. This could be attributed to the harsh environment (vineyards) from which our natural isolates were sourced as *GRE3* is reported to be stress-induced by heat shock, heavy metals, ionic and osmotic stress, oxidative stress, and starvation (Masuda e*t al.,* 2008). The environmental selective pressures from the vineyards where these strains were isolated could have led to higher baseline expression of *GRE3* which has been reported to display higher enzyme activity than overexpressed *S.s.xyl1* in some strains (Kogje and Ghosalkar, 2016; Konishi *et al.,* 2015).

As previously mentioned, the distinguishable xylitol titres generated by the different strains suggests that the genetics of the host organism had an influence over cell-specific productivities (Idiris e*t al.,* 2010). Apart from *GRE3,* natural *S. cerevisiae* strains harbour additional endogenous xylose-assimilating genes such as *SOR1* (sorbitol dehydrogenase) and *XKS1* (xylulose kinase) (Konishi *et al.,* 2015). Sorbitol dehydrogenase is homologous to xylitol dehydrogenase (XDH) in that it catalyzes the oxidation of xylitol to D-xylulose, while *XKS1* catalyzes the phosphorylation of D-xylulose (Ko *et al.,* 2006; Pantiño *et al.,* 2019; Pival e*t al.,* 2011). Although these genes are not adequately expressed to enable *S. cerevisiae* to naturally utilise xylose, they could lead to the assimilation of xylitol (Yang *et al.,* 2020b; Zha *et al.,* 2021).

Therefore, to increase xylitol yields, Yang *et al.* (2020b) investigated the deletion of *SOR1* or *XKS1* in an *S. cerevisiae* strain engineered to overexpress a heterologous XR gene. They reported that the deletion of the *XKS1* gene weakened *SOR1* expression which resulted in an accumulation of xylitol. The authors concluded that the industrial demands for xylitol production from lignocellulosic biomass could be met by deleting the *XKS1* gene. This strategy will be tested in our strains in future.

The co-fermentation strategy for xylitol production is essential as glucose provides the necessary reducing equivalents, supports cell growth, and maximises xylitol production from xylose (Narisetty *et al.,* 2021). The co-consumption of both sugar substrates is necessary to lessen fermentation time and attain productivity suited to industrial and economically viable processes (Moysés *et al*., 2016). However, glucose represses the intake and catabolism of alternate carbon sources which results in diauxic growth and the bi-phasic consumption of sugar where glucose is rapidly assimilated before other carbon sources are slowly consumed (Farwick *et al.,* 2014; Simpson-Lavy and Kupiec, 2019). To overcome this, Jo *et al.* (2015) made use of a glucose-limited fed-batch culture for xylitol production which resulted in high yields and productivities of xylitol from xylose. It has been reported that glucose to xylose ratios more than 1:10 negatively impact xylose transport and inhibit XR ability while a ratio less than or equal to 1:10 can enhance the XR action (López-Linares *et al.,* 2020; Tochampa *et al.,* 2005). In agreement with this, Narisetty e*t al.* (2021) reported that a glucose to xylose ratio of 1:10 was optimal for xylitol accumulation. Despite these reports, all of our engineered strain isolates produced similar or greater xylitol titres when cultured on the 1:1 instead of the 1:10 glucose to C5-sugar ratio (Figure 3.3.2 and Appendix Figure S4 and S5). However, the benefit of the 1:10 ratio may not have been properly utilised in this study due to the small-scale nature of our experimental set-up (5-10 mL). As mentioned above, glucose metabolism generates the NADPH required for xylose reduction and is needed to support cell growth (He *et al.,* 2021; Narisetty e*t al.,* 2021). Since only 0.2 % of glucose was used as a co-substrate in cultures employing the 1:10 ratio, insufficient glucose may have been available for cell growth and co-factor production. This may explain why some background strains cultured on the 1:1 glucose to pentose ratio produced higher xylitol titres than the engineered strains cultured using the 1:10 ratio (Figure 3.3.2 and Figure S5.a).

Furthermore, a rate-limiting step of xylose reduction is NAD(P)H regeneration (Jang *et al.,* 2003). As mentioned previously, glucose metabolism generates the NADPH required for xylose reduction (He *et al.,* 2021). As a result, researchers have made use of glucose-limited fed batch systems to allow continuous regeneration of NADPH for the activity of XR necessary for xylitol production (Jo *et al.,* 2015; Kogje and Ghosalkar, 2016). Our experiments were, however, conducted utilising batch instead of fed-batch fermentations. Despite this, our results are still surprising as the engineered strains were expected to perform significantly better since *S. stipitis* XR advantageously utilises both NADPH and NADH cofactors for xylitol production while *GRE3* is NADPH-dependent (Jo e*t al.,* 2015; Ruchala *et al.,* 2019; Träff-Bjerre *et al.,* 2004). This again points to the likelihood of our background strains harbouring highly active *GRE3* genes or the inefficient expression of the *S.s.xyl1* in our strains.

Table 3.1. Conversion of 20 g/L xylose to xylitol in strains cultured for 72 hours at 30 °C. All media was supplemented with 2 % glucose as a co-substrate. Values are presented as averages of biological triplicates and standard deviations are shown.

Strain isolate	Xylitol concentration (mg/L)	Conversion (%)
FIN1-X3	114	0,568 ± 0,094
FIN1-Cas9	104	0,520 ± 0,081
YI13-X3	410	2,049 ± 0,053
YI13-Cas9	324	1,622 ± 0,178
YI59-X3	223	1,113 ± 0,152
YI59-Cas9	205	1,023 ± 0,068

The low conversion rates presented in Table 3.1 are likely due to the culture conditions utilised in the study and the lower than expected XR production. Accordingly, culture conditions should be adjusted to optimise the xylose flux towards xylitol as mentioned above. Further genetic engineering to improve XR activity should also be applied. Therefore, in future we need to improve XR activity through a combination of strategies as discussed.

3.4 QUANTIFICATION OF XYLANASE AND XYLOSIDASE ENZYME ACTIVITY

Xylanases and β-D-xylosidases are the most essential enzymes required for xylan degradation and have been reported to act synergistically in hydrolysing xylan (Terrasan *et al.,* 2016). Xylanase activities of engineered and background strains were quantified by cultivating strains in YPD for 72 hours at 30 °C and analysing the culture supernatant in a DNS-based assay. Total cell cultures of the same cultivated strains were assayed using a pNP-X-based method to determine xylosidase activity. The heterologous xylanase and xylosidase activities, quantified as units per gram DCW (U/gDCW), are illustrated in Figure 3.4. The δ-integration method coupled with CRISPR-Cas9 successfully conferred *T.r.xyn2* xylanase and *P.t.r.xln43_SED1* xylosidase activity to our three strains (FIN1-X3, YI13-X3 and YI59-X3) as shown in Figures 3.4 a and b. The absence of enzyme activity in the host strains (FIN1-Cas9, YI13-Cas9 and YI59-Cas9) in both graphs indicated their lack of inherent xylanase and xylosidase activity.

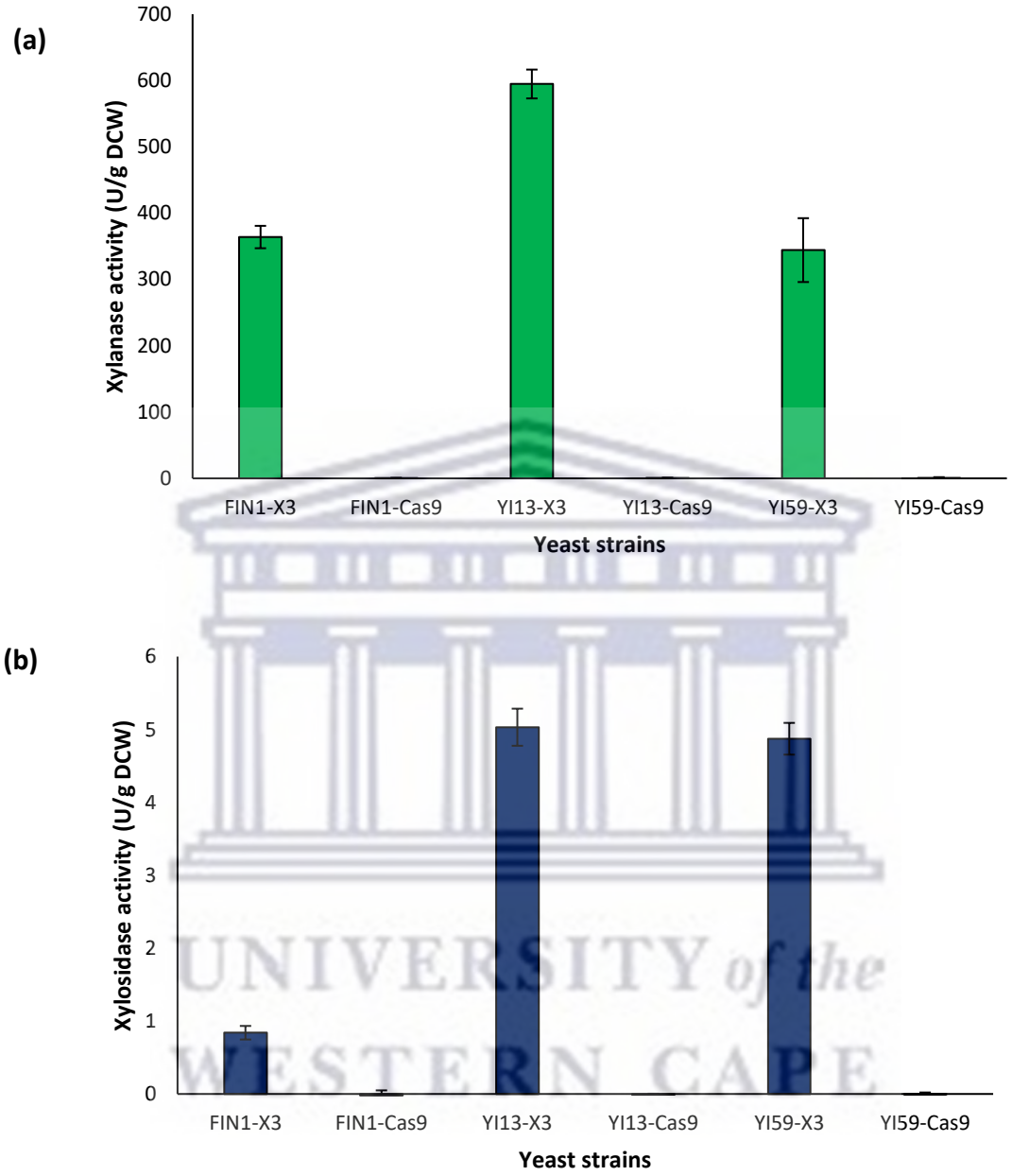

Figure 3.4. Enzyme activity profiles of metabolically engineered (-X3) and background (-Cas9) strains after 72h cultivation at 30 °C. (a) Xylanase and (b) xylosidase activities of engineered and background *S. cerevisiae* strains. Enzyme activities are expressed as units per gram DCW (U/g DCW) and are shown as averages of the enzyme assays performed in triplicate with error bars representing standard deviations.

All transformed strains exhibited high xylanase activity as depicted by Figure 3.4a. YI13-X3 was the top-performing xylanase strain with an activity of 595 U/gDCW. This was expected as YI13 has been reported to display a high innate secretory phenotype (Davison *et al.,* 2016). The unpaired t-test revealed that the xylanase activities for both FIN1-X3 and YI59-X3 were not

statistically different ($p \geq 0.05$). This was unexpected as FIN1 was previously reported to have a medium innate secretion capacity while YI59 had a low innate secretion capacity (Davison *et al.,* 2019). Nonetheless, the xylanase activities of both strains were quite substantial with FIN1-X3 having an activity of 364 U/gDCW and YI59-X3 having an activity of 344 U/gDCW. Katahira e*t al*. (2004) engineered a strain to co-display cell-attached *T.r.xyn2* and β-xylosidase from *Aspergillus oryzae* and reported a xylanase activity of 16 U/gDCW using birchwood xylan as a substrate. Kruger and Den Haan (2022) engineered a xylose-utilising laboratory strain to co-express *P.t.r.xln43_SED1* and secreted *T.r.xyn2* and recorded a xylanase activity of 26.3 U/gDCW when grown on glucose. Our obtained xylanase activities were significantly higher than these results. This could be attributed to our use of natural *S. cerevisiae* strains as hosts rather than domesticated strains. Davison *et al.* (2016) screened the heterologous cellulase activities in natural *S.cerevisiae* isolates and reported high enzyme secretory capacities among natural isolates as opposed to domesticated strains. Gronchi *et al.* (2022) engineered a natural *S.cerevisiae* strain, L20, alongside the benchmark yeast, Ethanol Red, to display amylolytic activity. They reported that L20 exhibited a significantly higher amylolytic activity than the industrial strain, despite having equal gene copies. These results confirm the strain-specific nature of heterologous protein secretion that has been reported numerous times before (Cho *et al.,* 2022; Davison *et al.,* 2016; Kastberg *et al.,* 2022; Minnaar and Den Haan, 2023).

Xylosidase activity was conferred to all transformed strains (FIN1-X3, YI13-X3 and YI59-X3) as indicated by Figure 3.4 b. Yet again, YI13-X3 was among the strains producing the highest heterologous enzyme activity with a xylosidase activity of 5 U/gDCW. However, an unpaired t-test proved that this xylosidase activity was not statistically different from that produced by YI59-X3 which had a xylosidase activity of 4.9 U/gDCW. FIN1-X3 showed the lowest xylosidase activity of 0.8 U/gDCW. With the exception of FIN1-X3, our results were more than double the xylosidase activity of the strain constructed by Kruger and Den Haan (2022) utilising glucose as a substrate (2.1 U/gDCW). These comparatively high xylosidase values could be attributed to the improved strength of the *SED1* promoter and *SED1* anchoring domain used to confer xylosidase activity to our host strains. Inokuma *et al.* (2014) reported that the simultaneous employment of the *SED1* promoter and anchoring domain enabled highly efficient enzyme incorporation into the cell walls of recombinant yeast strains. The lower xylosidase activity

observed in FIN1-X3 could have been influenced by the compatibility factor concerning the properties of the host cell and anchored xylosidase itself (Kroukamp *et al*., 2013; Van Zyl *et al*., 2014). The noticeable difference in xylanase and xylosidase activity demonstrates that the nature of the overexpressed heterologous protein affects the polypeptide production rate in host strains (Korpys-Woźniak *et al.,* 2020).

Additionally, the substantial heterologous xylanase and xylosidase activities could be credited to the overexpression of the two enzymes via CRISPR-Cas9-mediated multicopy δ-integration (Figure 1.8). The highly repetitive nature of the Ty delta elements dispersed throughout the *S. cerevisiae* genome enables these δ sequences to be exploited as target sites for an efficient multi-copy, multiple loci integration and stable expression of desired genes (Malci *et al.,* 2020; Song *et al.,* 2017). Furthermore, the disruption of transposable elements aids the production of strains with improved genetic stability (Malci *et al.,* 2020).

3.5 XYLITOL PRODUCTION FROM DIFFERENT C5-SUGAR SOURCES

The absence of an available source of economically feasible, pure xylose is a shortcoming in the industrial production of xylitol (Cheng *et al.,* 2014). Fortunately, biotechnological xylitol production does not exclusively hinge on purified xylose substrates (Chandel *et al.,* 2018). Biomass fractions rich in pentosans are promising sources for xylitol production (Irmak *et al.,* 2017). As such we set out to quantify the xylitol produced by our engineered strains using xylan, which is mainly composed of pentose sugars, as well as its depolymerised product (xylo-oligosaccharides) (Akpinar *et al.,* 2009; Smith *et al*., 2017). Beechwood xylan was the chosen source of xylan substrate due to its commercial availability and high xylose content (≥ 90 %) (Nieto-Domínguez *et al*., 2019). Figure 3.5 below depicts the different xylitol titres produced by our engineered and background strains from different C5-sugar substrates. As with section 3.3, we assayed xylitol production under different cultivation conditions (Appendix Figures S4 and S5). Again, the rates of conversion to xylitol were calculated and the values are presented in Tables 3.2a, b and c.

As seen by the three graphs of Figure 3.5 (a, b, and c), all the engineered yeast strains produced xylitol titres that far outperform that produced by the background strains. This

confirms the success of the metabolic engineering implemented for xylitol production from xylan and xylo-oligosaccharides.

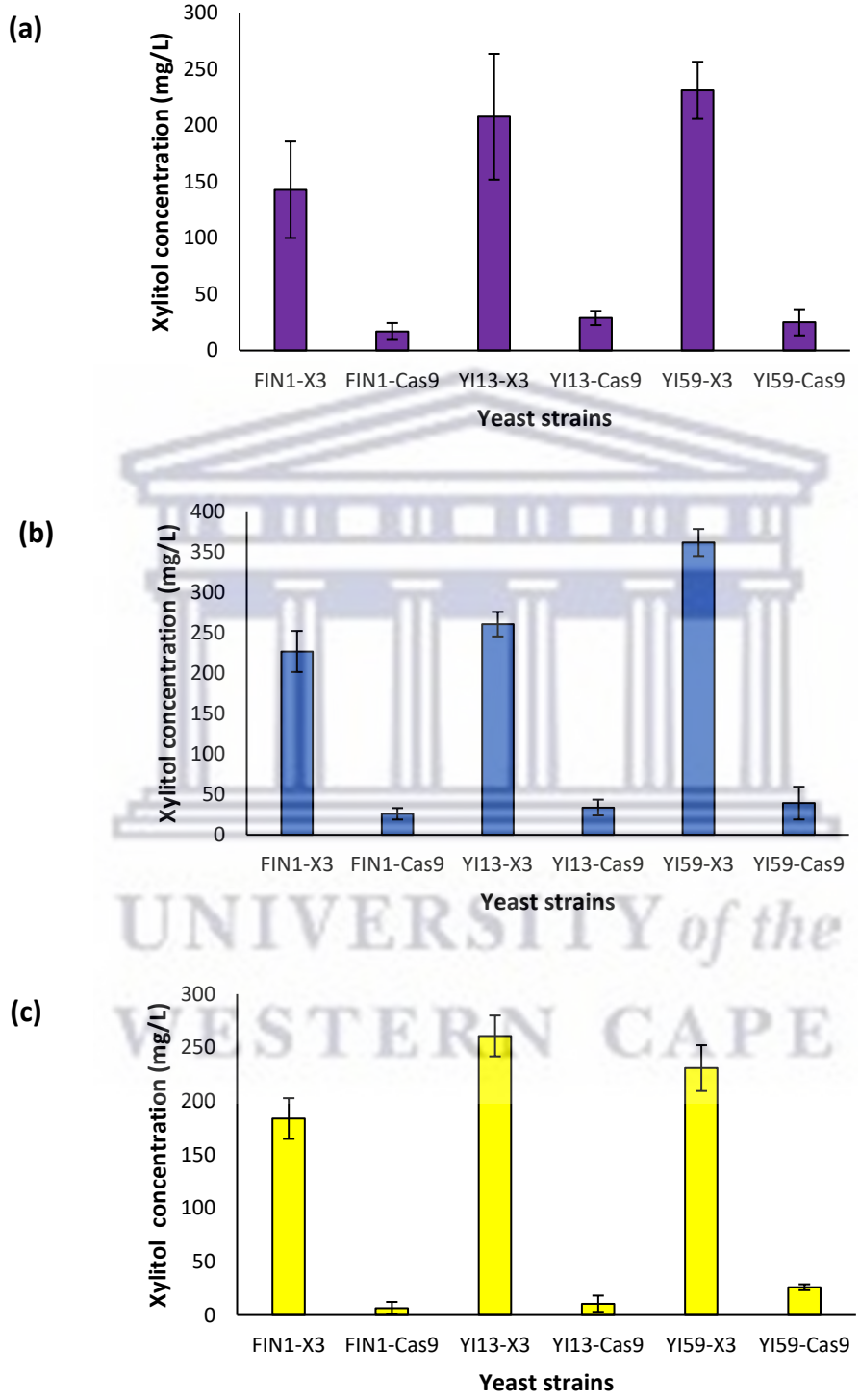

Figure 3.5. Xylitol production of metabolically engineered (-X3) and background (-Cas9) strains after 72h of cultivation on different C5 substrates at 30 °C. Xylitol titres produced by strains cultured on (a) 2 % xylan, (b) 2% xylo-oligosaccharides, and (c) both 2 % xylan and 2 % xylo-oligosaccharides. All media was supplemented with 2% glucose as a co-substrate. Xylitol concentrations are expressed as mg/L and are shown as averages of the enzyme assays performed in triplicate with error bars representing standard deviations.

Xylan degradation to xylose was achieved through the synergistic activity of *T.r.*XYN2 and *P.t.r.*XLN43_SED1: the endo-1,4-β-xylanase randomly hydrolysed the xylan backbone to produce XOS and some xylose residues allowing the β-xylosidase to continue the degradation of XOS into xylose (Li *et al*., 2021). After which, XR reduced the released xylose to xylitol. FIN1-X3, YI13-X3 and YI59-X3 produced an average of 143 mg/L, 208 mg/L, and 231 mg/L xylitol, respectively from 2 % xylan (Figure 3.5a). According to the unpaired t-test, the mean concentrations of xylitol produced by all three engineered strains from 2% xylan were not statistically different from each other. However, the xylitol titres produced by our engineered strains were considerably greater than that produced by the background strains.

The increased xylitol titres are quite noticeable in Figure 3.5b with FIN1-X3, YI13-X3 and YI59-X3 having produced an average of 227 mg/L, 260 mg/L and 361 mg/L, respectively. This was most likely attributed to the affinities of *T.r.*XYN2 and *P.t.r.*XLN43_SED1 for the xylo-oligosaccharide substrate. Enzymes belonging to the GH11 family (e.g. *T.r.*XYN2) have been reported to work best on long-chain XOS, cleaving the β-1,4-D-xylosidic bonds to produce mainly xylotriose and xylobiose (Li *et al.,* 2021; Procópio *et al.,* 2022; Thirametoakkhara *et al*., 2023). Degradation to xylose was continued by β-xylosidase which prefers xylobiose while its affinity for the XOS substrate rapidly decreases with increasing chain length (Procópio *et al.,* 2022). Xylose monomers were then reduced to xylitol by *S.s*.XYL1 and native GRE3 XR activity. Additionally, the greater xylitol concentrations produced from the 2 % xylo-oligosaccharide substrate could be attributed to the GH43 β-xylosidase coupled to the *S. cerevisiae* SED1 anchoring domain, expressed under the *SED1* promoter. The *P. tritici-repentis* GH43 β-xylosidase activity within the engineered strains is known to be a highly active β-xylosidase, efficiently displayed on the surface of our engineered cells via the simultaneous use of the *SED1* promoter and the stress-induced GPI-cell wall glycoprotein (Inokuma *et al.,* 2014; Jordan *et al.,* 2012, Kruger and Den Haan, 2022).

In Figure 3.5b, YI59-X3 was the top-producing strain (361 mg/L), agreeing with the xylosidase assay results of Figure 3.4b where YI59-X3 was among the top-performing isolates. The average xylitol concentrations produced by FIN1-X3 and YI13-X3 were not statistically different according to the unpaired t-test. Once again, the xylitol titres produced by the engineered strains were considerably greater than that produced by the background strains.

Figure 3.5c shows that FIN1-X3, YI13-X3 and YI59-X3 produced an average of 184 mg/L, 261 mg/L, and 231 mg/L xylitol, respectively from the 2 % xylan and 2 % XOS combination. For the most part, the comparison of Figures 3.5a, b, and c, infers that the xylitol production route incorporated into our natural yeast isolates utilized XOS better than it did xylan. All engineered strains produced among their highest average xylitol titres from the 2 % XOS substrate. The lowest xylitol concentrations were mainly generated from the 2 % xylan substrate. This is most likely due to the fact that, apart from hydrolysing the xylan backbone into XOS of varying degrees of polymerisation (DPs), GH11 xylanases are able to cleave long-chain XOS into the shorter chain xylo-oligomers preferred by xylosidases (Díaz-Arenas *et al.,* 2022; Huang *et al.,* 2017; Zhang *et al.,* 2022). The xylan substrate utilised – glucuronoxylan – also affected the results; the full conversion of the xylan to xylose was blocked by the glucuronic acid side chains present in the substrate (Maehara *et al.,* 2018). As observed in Figures 3.5a, b and c, no single strain outperformed the others despite them being reported to display different levels of enzyme secretory capacity (Davison *et al.,* 2019). This suggests that the low XR activity in the strains was hindering xylitol production. An alternate postulation could be that there was a feedback inhibition.

All background strains displayed in Figure 3.5 were able to assimilate some of the xylan and XOS substrates, likely due to some free xylose available in these pentose sources. The background strains' lack of inherent xylanase and xylosidase activity was also proven by Figures 3.4a and b. As mentioned, the endogenous non-specific aldose reductase encoded by *GRE3,* in *S. cerevisiae* is able to reduce xylose to xylitol (Masuda *et al.,* 2008; Träff *et al*., 2001). Li *et al.* (2015) reported that fungal xylose reductases are also able to reduce xylodextrins (xylosides and xylo-oligosaccharides) to oligomers of xylosyl-xylitol. Similarly, the native GRE3 enzyme could have reduced our XOS substrates to xylitol oligomers (Cai e*t al.,* 2014; Qian *et al.,* 2003).

Table 3.2. Conversion of different 20 g/L C5-sugar substrates to xylitol in strains cultured for 72 hours at 30 °C. (a) 20 g/L xylan converted to xylitol, (b) 20 g/L xylo-oligosaccharides converted to xylitol, (c) 20 g/L xylan and 20 g/L xylo-oligosaccharides converted to xylitol. All media was supplemented with 2 % glucose as a co-substrate. Values are presented as averages of biological triplicates and standard deviations are shown.

(a)

Strain isolate	Xylitol concentration (mg/L)	Conversion (%)
FIN1-X3	143	0.715 ± 0.214
FIN1-Cas9	17	0.085 ± 0.038
YI13-X3	208	1.039 ± 0.280
YI13-Cas9	29	0.145 ± 0.031
YI59-X3	231	1.157 ± 0.127
YI59-Cas9	25	0.126 ± 0.058

(b)

Strain isolate	Xylitol concentration (mg/L)	Conversion (%)
FIN1-X3	227	1.134 ± 0.127
FIN1-Cas9	26	0.130 ± 0.035
YI13-X3	260	1.302 ± 0.076
YI13-Cas9	34	0.168 ± 0.049
YI59-X3	361	1.807 ± 0.084
YI59-Cas9	39	0.196 ± 0.101

(c)

Strain isolate	Xylitol concentration (mg/L)	Conversion (%)
FIN1-X3	184	0.918 ± 0.095
FIN1-Cas9	6	0.032 ± 0.029
YI13-X3	261	1.304 ± 0.095
YI13-Cas9	11	0.053 ± 0.038
YI59-X3	231	1.154 ± 0.107
YI59-Cas9	26	0.130 ± 0.014

The vast difference in xylitol titres produced by the engineered and background strains in Figure 3.5 was quite encouraging, however the data presented in Tables 3.2 highlighted the low levels of conversion carried out by the strain isolates. Despite our engineered strains harbouring highly active xylanase and xylosidase activities (Figure 3.4), the conversion

efficiencies to xylitol were still exceptionally low with conversion rates peaking at 1.807 % (Table 3.2b; YI59-X3 on 2 % xylo-oligosaccharides). Lower levels of conversion were to be expected for strains cultivated on polymeric substrates owing to the side chains on glucoronoxylan affecting degradation (Najjarzadeh *et al.,* 2020). These values, however, were still lower than expected. Surprisingly, FIN1-X3 and YI59-X3 demonstrated higher levels of conversion to xylitol from xylan, XOS or both, than from pure xylose. Although this is promising for industry given the low availability of economically feasible pure xylose, the conversion rates must still be improved (Cheng *et al.,* 2014).

3.6 VALIDATION OF NATURAL ROBUSTNESS IN STRAIN ISOLATES

The well-established 1G bioethanol industry relied on existing knowledge from high gravity (HG) beer brewing and spirit distilleries for industrial strain development (Favaro *et al.,* 2019; Walker and Walker, 2018). However, the 2G biorefinery industry exposes yeast to different settings and several unique stress challenges (Favaro *et al.,* 2019). Such stress includes pretreatment-derived inhibitors that are cytotoxic and hinder metabolism, microbial growth, and the fermentation process (Paes *et al.,* 2021; Radecka *et al.,* 2015). Pretreatment of hemicellulose generates acetic acid (Chen *et al.,* 2012). Acetic acid is also a common co-product of *S. cerevisiae* fermentation, therefore under physiological conditions, the cells consume acetate through its respiratory metabolism. However, high concentrations of extracellular acetate can be toxic, leading to cell aging and death (Giannattasio *et al*., 2013; Guaragnella and Bettiga, 2021).

Accordingly, one of the traits of industrially ideal CBP strains are tolerance to the many stressors associated with the industry (Brethauer and Studer, 2015; Radecka *et al.,* 2015). Thermotolerance is a desirable trait of a CBP strain since the optimal temperatures of hydrolytic enzymes are higher than the ideal temperature for *S. cerevisiae* fermentation. Fungal xylanases perform best at temperatures ranging from 40-60 °C while *S. cerevisiae* strains have an optimum fermentative temperature of 22-32 °C (Cunha *et al.,* 2020; Yalcin and Ozbas, 2008; Yang e*t al.,* 2019).

Several strategies, including strain adaptation, rational design approaches and reverse engineering, are employed to improve the general robustness of 2G industrial strains (Brandt

et al., 2021). However, the exploitation of naturally tolerant strains would be beneficial as it would minimise the genetic modification required, thereby easing the metabolic burden inflicted on the engineered strains (Favaro *et al.,* 2019). Natural *S. cerevisiae* isolates are thought to be a potential source of CBP candidate strains owing to their adaptations which enable them to thrive in their harsh environments, while laboratory and industrial strains lack these adaptations because of domestication (de Witt *et al.,* 2019). As such we performed stress plate assays to characterise thermo- and acetic acid tolerance in our natural strain isolates as well as determine the effects that the heterologous enzyme expression had on strain robustness (Figure 3.6).

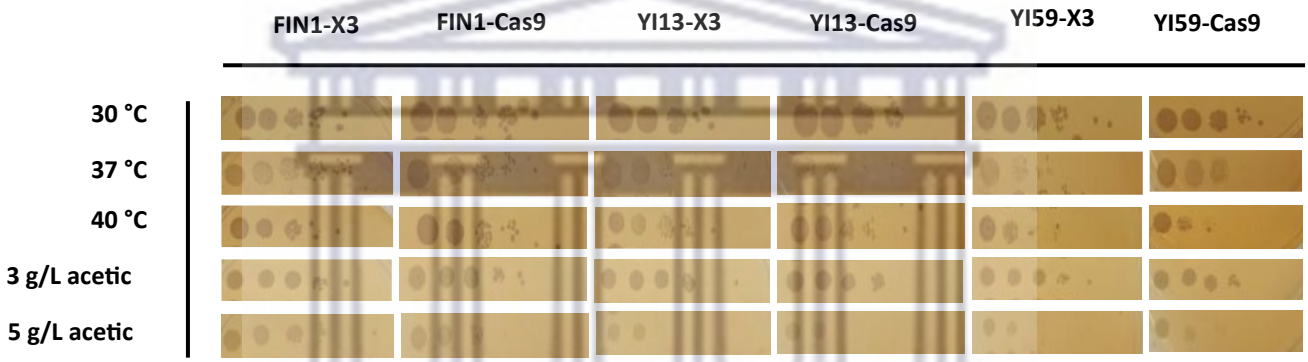

Figure 3.6. Characterisation of heat (30 °C, 37 °C, 40 °C) and acetic acid (3 g/L, 5 g/L) tolerance in metabolically engineered (-X3) and background (-Cas9) strains. 10-fold serial dilutions were spotted from left to right on YPD agar plates with the appropriate inhibitor and incubated at 30 °C unless otherwise stated. The starting dilution had an optical density of OD_{600nm} = 1. The stress plate assays were performed in triplicate and a representation of the average growth is shown.

Our engineered and background strains were characterised for robustness to varying temperatures (30 °C, 37 °C, and 40 °C). Both the engineered and background strains exhibited similar robustness when exposed to 30 °C - the optimal temperature of *S. cerevisiae* growth (Yalcin and Ozbas, 2008). Similar growth patterns are observed for the FIN1 strains grown at 37 °C, however the increased temperature clearly reduced the growth in the YI13 and YI59 strains, despite it being reported that YI13 displayed tolerance to high temperatures (Favaro *et al.,* 2019). Additionally, the metabolic burden imposed on YI59 seems to have reduced its robustness at 37 °C as indicated by the reduced growth in the transformed strain. Surprisingly, when the temperature was in range for ideal xylanolytic activity (40 °C), the FIN1 and YI13 strains exhibit growth similar to that displayed at 30 °C, while YI59 exhibits less tolerance (Yang

et *al.,* 2019). These results indicate that both FIN1 and YI13 natural isolates displayed tolerance to high temperatures while YI59 was a less tolerant strain, functioning best at 30 °C.

All strains exhibited high tolerance to 3 g/L acetic acid, and this could be attributed to the coastal and winery regions from which our strains were sourced (Davison *et al.,* 2016; Davison *et al.,* 2019). However, the growth of all strains drastically decreased upon exposure to 5 g/L acetate except for FIN1-X3. The results of acetic acid tolerance have demonstrated that the strains used in this study could possibly tolerate concentrations higher than 3 g/L but lower than 5 g/L. For the most part, Figure 3.6 indicates that the high-level heterologous protein expression did not affect the natural strains' tolerance to high temperatures and acetic acid concentrations. This supports the notion that the exploitation of natural isolates is advantageous as a CBP strain for 2G industrial processes (Davison e*t al.,* 2020).

3.7 GROWTH ANALYSIS

Heterologous protein production in host cells expends cellular resources that would normally be used for routine cellular processes, thus creating a competition for resources. This competition burdens host cells, negatively impacts cell fitness, may elicit stress responses, and often reduces the final protein concentrations (Kastberg *et al*., 2022). Furthermore, the overexpression of heterologous proteins exerts acute stress on host cells, thereby restricting the potential secretion yield (Davison *et al.,* 2019). La Grange e*t al.* (2001) reported a reduction in cell yields when both heterologous β-xylanase and β-xylosidase were highly expressed and they accredited it to the metabolic burden brought about by this high-level expression.

Not only have the strains used in this study been metabolically engineered to overexpress three heterologous enzymes but they were also constitutively expressed. We, therefore set out to determine the metabolic burden experienced by our engineered strains by analysing and comparing the growth patterns of each engineered strain isolate to their respective host strain, until stationary phase was reached. The growth patterns of each strain are shown in Figure 3.7.

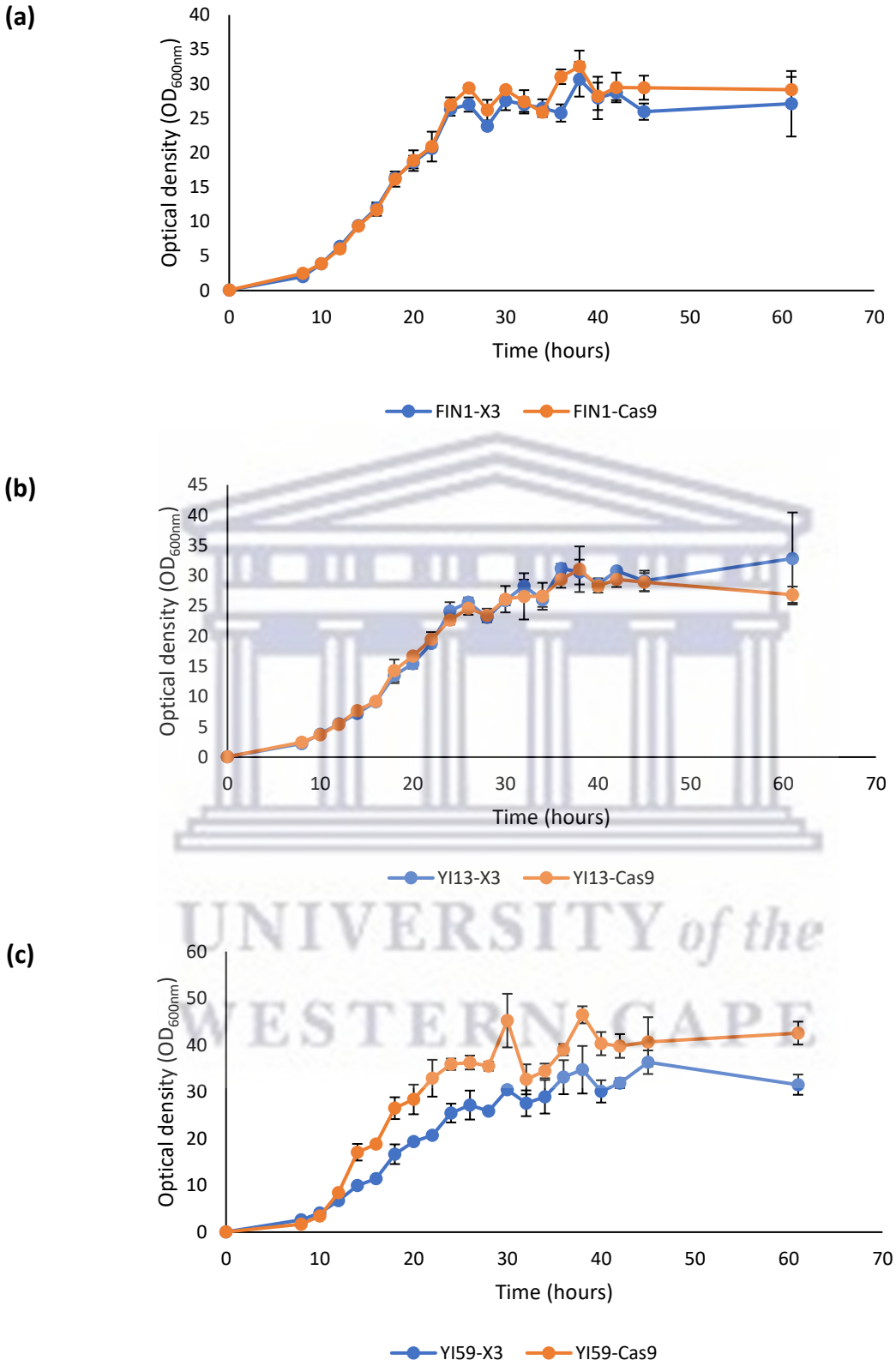

Figure 3.7. Growth curves of engineered and background (a) FIN1, (b) YI13 and (c) YI59 strains grown on YPD at 30 °C for 61 hours. Values are displayed as the averages of three biological samples per strain isolate with error bars representing standard deviations.

Comparison of the growth in Figure 3.7, displayed no significant variation between the engineered and respective background strains of FIN1 and YI13, despite the engineered strains harbouring constitutively, overexpressed industrial enzymes. However, this was not the case for the YI59-based strains; the engineered strain (YI59-X3) displayed noticeable reduced levels of growth compared to YI59-Cas9. The growth of YI59-Cas9 peaked at OD_{600nm} = 46.47, contrasted to the growth peak of the engineered YI59-X3 which was recorded at OD_{600nm} = 36.33 (Figure 3.7c). Similarly, Minnaar and Den Haan, (2023) reported that their engineered YI59-based strain exhibited significantly impaired growth compared to its untransformed counterparts. The authors assumed that the high enzyme activity levels produced, meant that greater protein production levels were expressed to the detriment of biomass production. It was surprising that this difference in growth patterns was only observed for the engineered and background YI59 strain isolates when no single strain outperformed the other (Figure 3.5). Despite the noticeable difference in growth levels amongst the YI59 engineered and background strains, the low conversion efficiencies to xylitol cannot be credited to the undue metabolic burden of the engineered strains but more likely to the lower-than-expected xylose reductase activity present in our engineered *S. cerevisiae* strains. The findings presented in Figure 3.7 further support the notion of exploiting natural strain isolates in LCB biorefineries over domesticated isolates.

CHAPTER 4

SUMMARY AND CONCLUSION

To achieve a sustainable and economically viable 2G biofuels industry, biorefineries must co-produce high-value, low-volume bioproducts alongside high-volume, low-value cellulosic ethanol (Budzianowski, 2017; Tana *et al.,* 2021). Given the well-established xylitol market, this study aimed to engineer a xylitol-production route in three natural isolate diploid *S. cerevisiae* strains. This was achieved by introducing heterologous genes into the delta regions of yeast genomes via conventional and CRISPR-Cas9-based transformation strategies. Furthermore, strain tolerance to common 2G biorefinery-associated stressors were evaluated to confirm innate robustness reported in our natural strains. It was hoped that the engineering of a xylitol-producing natural yeast strain, would allow the manufacture of the high value co-product in a biorefinery.

We successfully transformed our natural strains with *P.tr.xln43_SED1*, *T.r.xyn2* and *S.s.xyl1*, encoding a β-xylosidase, β-xylanase and a xylose reductase. The overexpressed *S.s.xyl1* failed to improve on the xylose reduction ability conferred to our strains, likely by their native *GRE3* gene. However, xylitol production from 2 % xylan and XOS was successful and is valuable for the 2G biorefinery industry given the high cost of pure xylose. The high xylanase and xylosidase activities exhibited by our engineered strains supported the notion that utilising natural yeast strains as bioengineering hosts served as feasible, superior starting points. This notion was further supported by analysing the innate tolerance and growth patterns of the engineered and background strains. All engineered strains displayed tolerance to acetic acid concentrations higher than 3 g/L but lower than 5 g/L while FIN1-X3 and YI13-X3 displayed tolerance to temperatures as high as 40 °C. Growth analyses revealed that only YI59-X3 displayed somewhat impaired growth, however, no single strain outperformed the other across the recorded assays of this study. The results of this study led us to conclude that the xylose reduction ability of these strains must be enhanced through alternate genetic engineering strategies on which we elaborate below. The engineering strategies employed for heterologous xylanase and xylosidase activity as well as the use of natural strains as bioengineering hosts, offer considerable potential for use in the 2G biorefinery industry.

4.1 FUTURE PROSPECTS

To fulfil the economic requirement of second generation biorefineries, the conversion efficiency of xylose to xylitol is top priority (Yang *et al*., 2020a). Therefore, future investigations should focus on genetic engineering strategies aimed at improving xylose reductase activity. The focus could be shifted from heterologous XR proteins to the native GRE3 enzyme; Kogje and Ghosalkar (2016) evaluated the xylitol activity in strains overexpressing both heterologous XR encoding genes and endogenous *GRE3* and reported that the strain overexpressing *GRE3* presented the best xylitol productivity. Therefore, given the high baseline expression of *GRE3* in our host strains, optimised strain engineering should include the overexpression of GRE3 in our host strains. To further increase xylitol production, the endogenous xylose-assimilating gene encoding xylulose kinase (*XKS1*) in *S. cerevisiae* strains should be deleted. Yang e*t al.* (2020b) reported an accumulation in xylitol production after deleting *XKS1* from *S. cerevisiae* strains. Alternatively, since *Candida* sp. are reported to be the most attractive xylitol producers, heterologous XR encoding genes from *Candida* sp. can be investigated (Singh *et al.,* 2023). Strains can also be improved by ensuring that genes are introduced in high copy numbers (Salazar-Cerezo *et al*., 2023).

Secreted expressed heterologous proteins in *S. cerevisiae* are frequently exposed to bottlenecks, limiting their yield. Culture conditions is one of the factors affecting the yield and as a result, literature offers many reports addressing the optimisation of fermentation processes (Barathikannan and Agastian, 2016; Cho *et al.,* 2022; He *et al.,* 2021; Jo *et al.,* 2015; Li *et al.,* 2013b). Therefore, as an alternate future prospect, the overexpressed heterologous *S.s.xyl1* activity could possibly be improved by optimising our culture conditions. The fermentation parameters can be improved by increasing the culture volume and conducting the experiment in a fed-batch system under glucose-limited conditions (in a ratio of 1 glucose to 10 xylose) to allow the continuous regeneration of NADPH necessary for xylose reduction. This experimental set-up resulted in high yields and productivities of xylitol from xylose (Jo *et al.,* 2015).

CHAPTER 5

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APPENDIX 1

SUPPLEMENTARY FIGURES AND TABLES

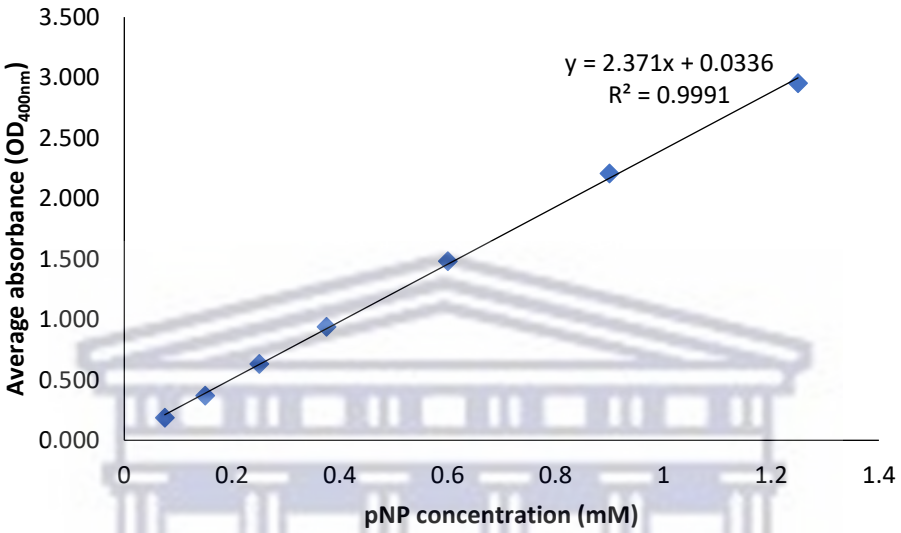

Figure S1. *p*-nitrophenyl (pNP) standard curve used to determine the amount of pNP liberated by the action of *P.t.r.*XLN43_SED1.

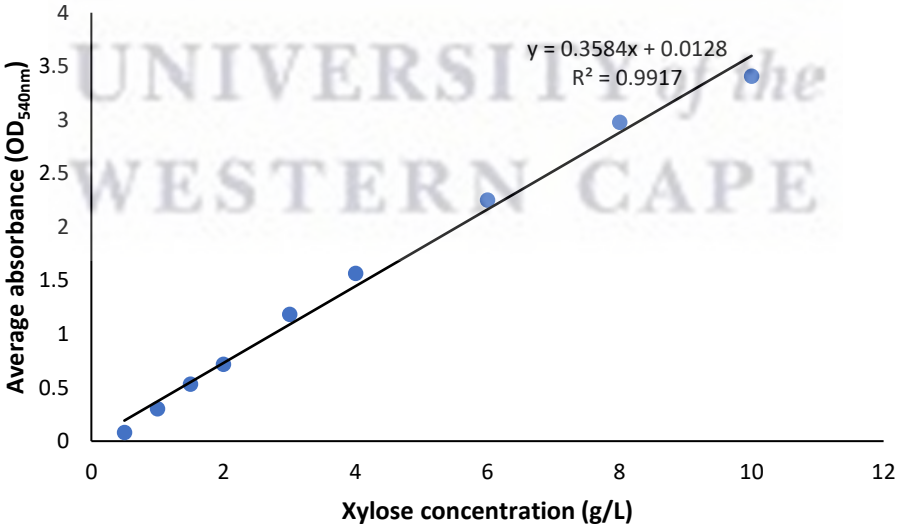

Figure S2. Xylose standard curve used to determine the amount of xylose liberated by the action of *T.r.*XYN2.

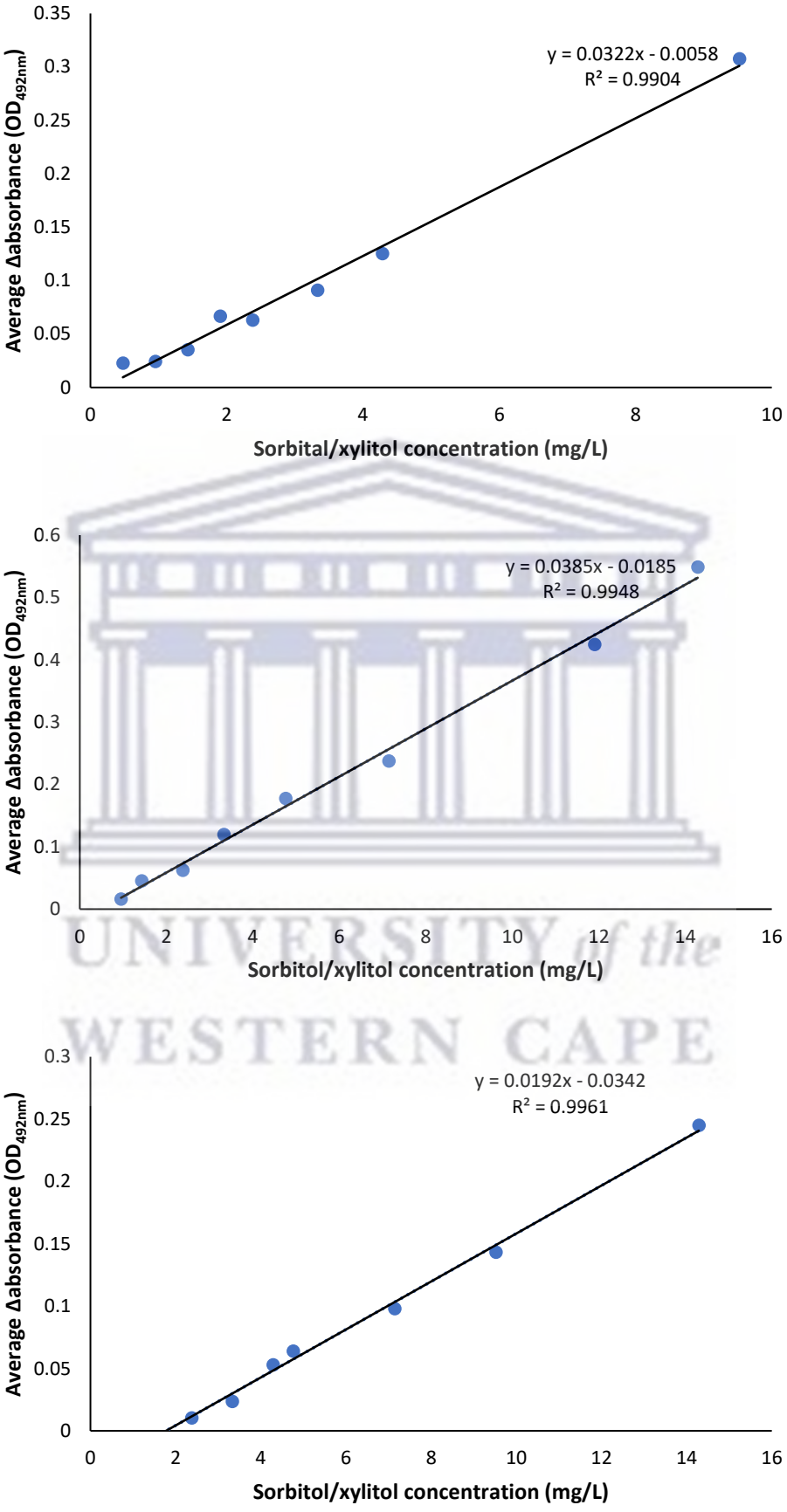

Figure S3. D-Sorbitol standard curves used to determine the amount of xylitol produced by the yeast strain isolates. A new standard curve was created with each batch of D-Sorbitol/Xylitol assay kit.

Table S1. Comparison of the XR activity in conventionally and CRISPR-Cas9-based transformed YI59 strains after growth until log phase at 30 °C. The enzymes were targeted for δ-integration in all strains. Values are presented as averages of biological triplicates and standard deviations are shown. TPE = total protein extract.

Strain and µL of TPE used	Transformation strategy	Enzyme activity (µmol/min.mL^{-1})
YI59 (10 µL)	conventional	0.063 ± 0.022
	CRISPR-Cas9	0.045 ± 0.008
YI59 (20 µL)	conventional	0.048 ± 0.006
	CRISPR-Cas9	0.035 ± 0.002

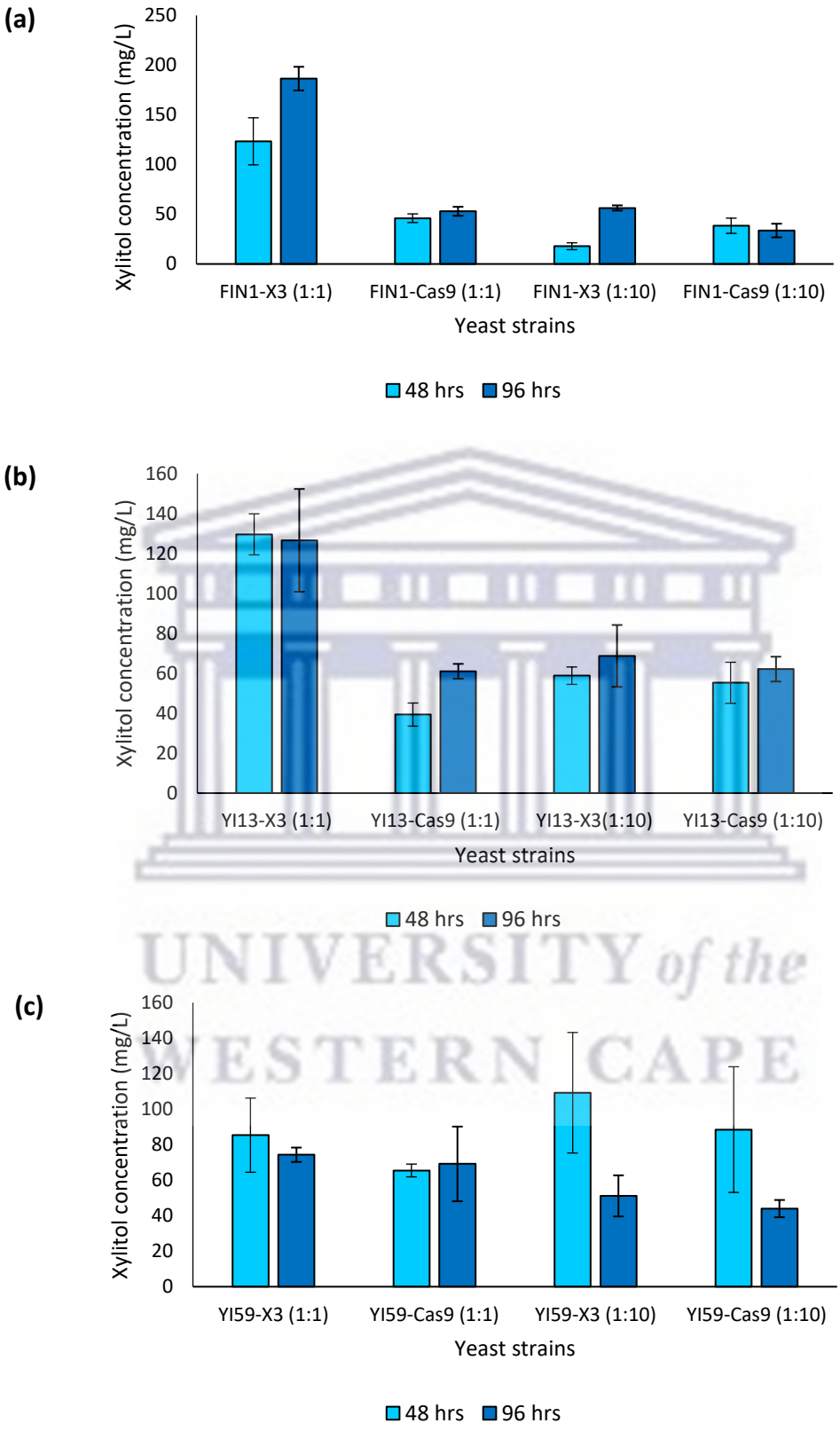

Figure S4. Xylitol production of metabolically engineered (-X3) and background (-Cas9) yeast strains after 48 and 96 h cultivations on 2 % xylo-oligosaccharides at 30 °C. Graphs (a), (b) and (c) illustrate the xylitol titres produced by FIN1, YI13 and YI59 strain isolates, respectively. The media was supplemented with either 2 % (1:1) or 0.2 % (1:10) glucose as a co-substrate. Xylitol concentrations are expressed as mg/L and are shown as averages of the enzyme assays performed in triplicate with error bars representing standard deviations.

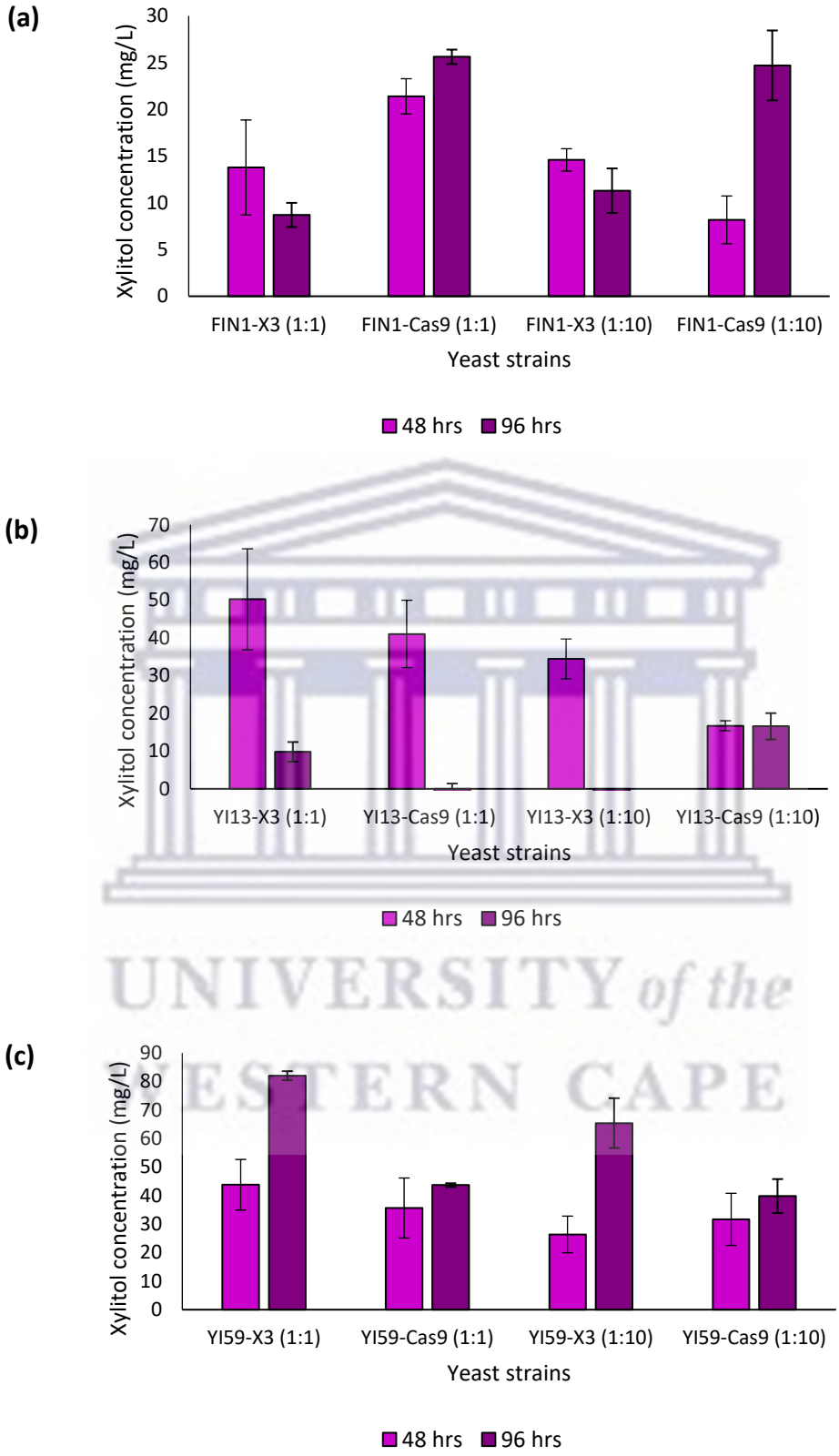

Figure S5. Xylitol production of metabolically engineered (-X3) and background (-Cas9) yeast strains after 48 and 96 h cultivations on 2 % xylan at 30 °C. Graphs (a), (b) and (c) illustrate the xylitol titres produced by FIN1, YI13 and YI59 strain isolates, respectively. The media was supplemented with either 2 % (1:1) or 0.2 % (1:10) glucose as a co-substrate. Xylitol concentrations are expressed as mg/L and are shown as averages of the enzyme assays performed in triplicate with error bars representing standard deviations.

Figure S4 generally depicts no significant difference between cultivation after 48 and 96 hours on 2 % XOS. The engineered strain isolates cultured on 2 % xylan (Figure S5) all displayed different levels of xylitol production over the two time points (48 and 96 hours); FIN1-X3 appeared to produce similar xylitol titres after 48 and 96 hours, surprisingly, YI13-X3 produced reduced xylitol titres after 96 hours, or no xylitol was detected after 96 hours, and YI59-X3 generated its highest xylitol concentrations after 96 hours. The values differ from those on the earlier Figures (Figure 3.5), likely because of batch variation between the kits used.

APPENDIX 2

ADDITIONAL DETAILS FOR CALCULATION OF NADPH OXIDATION

Beers Law: $(A = \varepsilon bc)$, A = absorbance, Ɛ of NADPH = 6 220 $M^{-1}.cm^{-1}$, b = 0.588 cm to determine concentration of NADPH.