EXPLORING NEW *SACCHAROMYCES CEREVISIAE* STRAINS SUITABLE FOR THE PRODUCTION OF CELLULOSIC BIOETHANOL

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DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

This dissertation includes one original paper published in a peer-reviewed journal and one unpublished publication. The development and writing of the papers (published and unpublished) were the principal responsibility of myself and, for each of the case where this is not so, a declaration is included in the dissertation indicating the nature and extent of the contributions from co-authors.

March 2019

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Declaration by the candidate

With regard to Chapters 1-6, the nature and scope of my contributions were as follows:

Nature of contribution	Extent of contribution
Planning and execution of the experimental work, data analysis, and preparation of draft manuscript	90 %

The following co-authors have contributed to Chapters 2-5

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Declaration by co-authors:

The undersigned hereby confirm that

1. the declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to Chapters 2 to 5;

2. no other authors contributed to Chapters 2 to 5, besides those specified above; and

3. potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in Chapters 2 to 5 of this dissertation.

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SUMMARY

Natural *Saccharomyces cerevisiae* strains display an enhanced robustness that is associated with the environment they occupy. This robustness is expressed through complex biological networks and provides the organism with the ability to maintain cell viability during adverse environmental conditions.

In this study, the natural diversity of *S. cerevisiae* was exploited to obtain strains with phenotypic characteristics beneficial for second-generation bioethanol production. Artificial hybridisation was employed to increase the genetic, and thus the phenotypic diversity of these strains, whereafter transcriptomics were utilised to elucidate the molecular mechanisms that allow adaptation to an increase in temperature and survival in an environment containing inhibitory compounds that are associated with the degradation of cellulosic feedstock.

Our results indicated that there is an association between strain diversity, the environment and the geographic location, whilst, individual strains display phenotypes. The Cape West Coastal region was associated with inhibitor-resistant strains, whereas the Breede River valley was characterised by inhibitor-sensitive strains. Strains that displayed an increased fermentation capacity were associated with the Cape South Coast. Several strains with tolerant phenotypes i.e. the ability to grow and/or ferment under a range of environmental conditions, were identified, including a multi-tolerant strain, YI13, with growth tolerance against ethanol (15 % v/v), inhibitors (15 %) and increased temperature (45 °C). Two inhibitor tolerant (25 %) strains, HR4 and YI30, displayed improved fermentation capacity (0.22 and 0.35 g/L/h) during aerobic and anaerobic conditions, respectively.

Artificial hybridisation generates genetic diversity that affects the phenotype of the organism and was applied to produce progeny strains. Several of these strains displayed inhibitor tolerance heterosis, whereas pH and salt tolerance decreased relative to the parental strains. In addition, unique phenotypes were generated, with strain HR4/YI30#6 displaying growth at 2 M NaCl and in 20 % ethanol. A single multi-tolerant strain, V3/YI30#6, with unique (2 M NaCl and 45 °C tolerance) and general (25 % inhibitor tolerance) traits was obtained. However, the fermentation capacity of this strain

was decreased to a theoretical ethanol yield of 60 % compared to ~80 % for the parental strains. This indicates that although hybridisation produces heterosis and novel phenotypes, there is a limit to the degree of phenotypic diversity that can be obtained in a single strain. This may be due to the high energy demand (due to the increase in metabolic flux of certain biological processes) during the various stress responses, whilst maintaining cell viability.

The molecular mechanisms for inhibitor and temperature tolerance of two natural strains were subsequently investigated. In addition to several biological processes, an upregulation of amino acid biosynthesis and ribosome biogenesis was observed in the temperature tolerant strain, YI13. This was possibly in response to irreversible protein damage inflicted by reactive oxygen species generated in response to an increase in temperature. The main contributor to inhibitor tolerance in strain YI30 was the activation of the oxidative stress response. This is probably due to the increased oxidoreductase activity required for the detoxification of the inhibitory compounds. Activation of the traditional heat shock response did not play a major role in combating temperature stress, however, an upregulation in this stress response was observed in reaction to inhibitor stress.

This study indicates that the natural diversity of *S. cerevisiae* yields unique strains and that phenotype diversity can be enhanced through hybridisation. In addition, *S. cerevisiae* strains display similar mechanisms in response to environmental stress. However, the specific molecular mechanisms that allow robustness and the degree to which these stress responses are activated, are strain dependent. A single strain will not be able to display all the required characteristics for a particular process, therefore a compromise will have to be made where the characteristics of the host organism and the specific application are considered, including genetic engineering of yeast strains.

OPSOMMING

Natuurlike *Saccharomyces cerevisiae* stamme toon 'n verhoogde robuustheid wat verband hou met die omgewing wat dit beset. Die robuustheid word deur komplekse biologiese netwerke uitgedruk en bied die organisme die vermoë om sel-lewensvatbaarheid tydens ongewenste omgewingsomstandighede te handhaaf.

In hierdie studie is die natuurlike diversiteit van *S. cerevisiae*-stamme uitgebuit om stamme met fenotipiese eienskappe te verkry wat voordelig vir die produksie van tweede generasie bio-etanol is. Kunsmatige hibridisering is gebruik om die genetiese en dus fenotipiese diversiteit te verhoog, waarna 'n transkriptomiese benadering gebruik is om die molekulêre meganismes te verklaar wat aanpassing by 'n verhoogde temperatuur en oorlewing in 'n omgewing wat inhiberende verbindings bevat, wat met die afbraak van sellulose-voer geassosieer is, toe laat.

Ons resultate het aangedui dat daar 'n verband tussen stamdiversiteit, die omgewing, en die geografiese ligging bestaan, terwyl individuele stamme unieke fenotipes vertoon. Die Kaapse Weskusstreek gebied was geassosieer met stamme wat 'n inhibeerder-weerstandbiedende fenotipe getoon het, terwyl die Breederiviervallei gekenmerk word deur inhibeerder-sensitiewe stamme. Stamme wat 'n verhoogde fermentasievermoë toon, is met die Kaapse Suidkusstreek geassosieer. Verskeie stamme met tolerante fenotipes dws, die vermoë om te groei en/of te fermenteer onder 'n verskeidenheid omgewingstoestande, is geïdentifiseer, insluitende 'n multi-tolerante stam, YI13, wat groeitoleransie teen etanol (15 % v/v), inhibitore (15 %) en temperatuur (45 °C) toon. Twee inhibitortolerante (25 %) stamme, HR4 en YI30, het verbeterde (0.22 en 0.35 g/L/) fermentasiekapasiteit gedurende onderskeidelik aërobiese en anaërobiese toestande getoon.

Kunsmatige hibridisering genereer genetiese diversiteit wat die fenotipe van die organisme beïnvloed en was gebruik om nageslagstamme te genereer. Verskeie van hierdie stamme het inhibitortoleransie heterosis getoon, terwyl pH- en souttoleransie afgeneem het. Daarbenewens is unieke fenotipes gegenereer, met HR4/YI30#6 wat groei in 2 M NaCl en in 20 % etanol getoon het. ‘n Enkele multi-tolerante stam, V3/YI30#6, met unieke (2 M NaCl en 45 °C toleransie) en algemene (25 % inhibitortoleransie) eienskappe,

is verkry. Die fermentasievermoë van hierdie stam het egter verlaag tot 'n teoretiese etanol opbrengs van 60 % in vergelyking met ~80 % vir die ouerstamme. Dit dui daarop dat alhoewel hibridisasie heterosis en nuwe fenotipes toelaat, daar 'n beperking op die mate van fenotipiese diversiteit is wat in 'n enkele stam verkry kan word. Dit kan wees as gevolg van die hoë energie behoefte (weens die toename in metaboliese fluks van sekere biologiese prosesse) tydens die verskillende stresreaksies terwyl sellewensvatbaarheid behoue moet bly.

Die molekulêre meganismes vir inhibitor- en temperatuurtoleransie van twee natuurlike stamme is vervolgens ondersoek. Benewens verskeie biologiese prosesse, is 'n opregulering van aminosuurbiosintese en ribosoombiogenese in die temperatuurtolerante stam YI13 waargeneem. Dit was moontlik in reaksie op die onomkeerbare proteïenskade wat veroorsaak is deur reaktiewe suurstofspesies wat gegenereer is in reaksie op ‘n toename in temperatuur. Die belangrikste bydrae tot inhibitortoleransie in stam YI30 was die aktivering van die oksidatiewe stresreaksie. Dit is waarskynlik te wyte aan die verhoogde oksidoreduktase aktiwiteit wat benodig word vir die detoksifisering van die inhiberende verbindings. Aktivering van die tradisionele hitte-skokreaksie het nie 'n belangrike rol in die bestryding van temperatuurstres gespeel nie, maar 'n opregulering in hierdie stresreaksie om inhibitorstres te hanteer, is waargeneem.

Hierdie studie dui daarop dat die natuurlike diversiteit van *S. cerevisiae* unieke stamme lewer en dat die fenotipese diversiteit deur hibridisasie verbeter kan word. Daarbenewens vertoon *S. cerevisiae* dieselfde meganismes om omgewings stres te hanteer. Die spesifieke molekulêre meganismes wat robuustheid toelaat, asook die mate waartoe hierdie stresreaksie geaktiveer word, is egter van die spesifieke stam afhanklik. Geen enkele stam sal aan al die vereiste eienskappe vir 'n bepaalde proses kan voldoen nie, dus moet 'n kompromie gevind word waar die eienskappe van beide die gasheerorganisme en die spesifieke toepassing oorweeg word, insluitende genetiese manipulasie van gisrasse.

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PREFACE

The dissertation is presented as a compilation of separately introduced scientific manuscripts. Sections of Chapter 3 and 4 were published in a single manuscript (Publication 1, attached as addendum). Chapter 5 is in preparation and is written in a style of the journal to which the manuscript will be submitted (Publication 2).

Chapter 3 and 4:	Mating of natural *Saccharomyces cerevisiae* strains for improved glucose fermentation and lignocellulosic inhibitor tolerance
	Published in Folia Microbiologica (2018) Vol 63:155-168
Chapter 5:	Transcriptomic analysis of gene induction in temperature and inhibitor tolerant natural *S. cerevisiae* strains
	In preparation for submission

To Nickey and Jaimé, for all the sacrifices you made to support me on this journey.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABC ATP-binding cassette

AFP Acids, furan aldehydes, phenols

ASE Allele-specific expression

CBP Consolidated bioprocessing

CNV Copy number variation

DEGs Differentially expressed genes

ER Endoplasmic reticulum

ESR Environmental stress response

GCN General control nonderepressible

GMO Genetically modified organism

GRAS Generally regarded as safe

GSR General stress response

GWAS Genome-wide association studies

GxE Genotype-by-environment

HGT Horizontal gene transfer

HMF 5-hydroxymethylfurfural

HOG high osmolarity glycerol

HSE Heat shock element

HSF Heat shock factor

HSP Heat shock protein

HSR Heat shock response

Indels Insertions and deletions

LCB Lignocellulosic biomass

MAPK Mitogen-activated protein kinase

MDR Multidrug resistance

MFS major facilitator superfamily

MMS Methyl methanesulphonate

MSN Multicopy suppressor of SNF1 mutation

NGS Next-generation sequencing

ORF Open reading frame

PACE Proteasome-associated control element

PDR Pleiotropic drug response

PDRE Pleiotropic drug response element

PKA Protein kinase A

PKC Protein kinase C

PPP Pentose phosphate pathway

QTL quantitative trait loci

RNA-seq RNA sequencing

RPN Regulatory particle non-ATPase

ROS Reactive oxygen species

SAR Structure-activity relationship

SCP Single cell protein

SDG Sustainable development goals

SHCF Separate hydrolysis, and co-fermentation

SHF Separate hydrolysis, and fermentation

SNP Single nucleotide polymorphism

snRNP small nuclear ribonucleoprotein

SSA Sub-Saharan Africa

SSCF Simultaneous saccharification, and co-fermentation

SSF Simultaneous saccharification, and fermentation

SSFF Simultaneous saccharification, filtration, and fermentation

STRE Stress response promoter element

TCA Tricarboxylic acid cycle

TF Transcription factor

UFA unsaturated fatty acid

UPP Ubiquitin-mediated proteasome degradation pathway

YAP Yeast activated protein

YRE Yeast activated protein response element

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CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

CHAPTER 1: GENERAL INTRODUCTION AND PROJECT AIMS

1.1 GENERAL INTRODUCTION

Saccharomyces cerevisiae is regarded as the model eukaryotic host for fundamental scientific discovery and biotechnological and industrial applications (Johnson and Echavarri-Erasun 2011). *Saccharomyces cerevisiae* has contributed to the understanding of biological processes in various disciplines including evolution and ecology (Replansky et al. 2008; Hittinger 2013), molecular genetics (Duina et al. 2014) and medicine and medical research (Mager and Winderickx 2005; Matuo et al. 2012). In addition, this organism has been used for centuries in the production of fermented foods and alcoholic beverages. This allowed for the accumulation of a vast knowledge base on *S. cerevisiae,* promoting its use in improving traditional industries (food, alcoholic beverages, feed, and agriculture), as well as in biotechnological applications, permitting its use in the pharmaceutical, medical and chemical industries (Johnson and Echavarri-Erasun 2011).

Strains of *S. cerevisiae* represent an ideal host due to their genomic diversity, phenotypic plasticity and amenability to genetic manipulation. Although industrial strains are diverse and display phenotypic plasticity, their diversity is limited when compared to natural strains (Steensels and Verstrepen 2014; Peter et al. 2018). There is a relationship between the geographic location, the environment and the genetic and phenotypic diversity displayed by strains of *S. cerevisiae* due to the genetic-environment interaction of *S. cerevisiae* (Liti et al. 2009; Strope et al. 2015; Peter et al. 2018). This robustness is further enhanced by the habitat diversity displayed by *S. cerevisiae* (Mortimer 2000; Camarasa et al. 2011). In addition, *S. cerevisiae* is able to elicit a variety of independent and integrated stress responses when exposed to unfavourable environments, which allows it to adapt to its environment (Gasch 2002; Morano et al. 2012; Minic 2015; Kawakami et al. 2016; Taymaz-Nikerel et al. 2016; Saini et al. 2018). Aside from geography and environment, reproduction allows for genome shuffling and therefore enhances the genetic diversity observed in *S. cerevisiae* (Peter et al. 2018). Genomic hybridisation (whether natural or artificial) can therefore be used to improve the diversity of this organism, thus increasing its application potential (Pulvirenti et al. 2002; Hashimoto et al. 2006; Inoue et al. 2014).

The drive towards social responsibility and sustainable living has promoted the use of alternative clean energy sources, of which biofuel is one of the most successful platforms currently used on an industrial scale (Guo et al. 2015). First-generation biofuel production with *S. cerevisiae* as a microbial host competes with food as a feedstock, undermining food security, especially in developing countries (Lynd et al. 2015). Second-generation biofuels that use cellulosic material as feedstock provide a suitable alternative to first-generation biofuel production, but current technologies make the production of this biofuel uneconomical (Lynd et al. 2005; Lynd et al. 2017). Consolidated bioprocessing presents a sustainable process for the production of renewable energy (Van Zyl et al. 2007). However, this process requires pretreatment, followed by simultaneous microbial hydrolysis and fermentation of the lignocellulosic biomass (Lynd et al. 2005). In order to achieve this, a robust microorganism is required that is multi-stress tolerant (inhibitor, temperature, pH, ethanol and osmotolerant) together with a high ethanol fermentation capacity.

The robustness and genetic amenability of *S. cerevisiae* along with the ability of this organism to retain viability in unfavourable environments, make it an ideal candidate for use in cellulosic bioethanol production due to the toxicity associated with this process. Natural South African *S. cerevisiae* strains have not been studied extensively (Liti et al. 2009; Strope et al. 2015; Peter et al. 2018) and therefore provide an unexplored source of potential strains for biotechnological and industrial applications, specifically in the production of cellulosic bioethanol.

1.2 AIMS OF THE STUDY

The aims of this study were (1) to evaluate the phenotypic landscape of natural *S. cerevisiae* strains; (2) to enhance the phenotypic profile of *S. cerevisiae* strains; and (3) to elucidate the cellular mechanisms involved in environmental stress tolerance in natural *S. cerevisiae* strains.

The objectives identified to achieve the aims of the study were:

1. To assess the physiological characteristics of natural *S. cerevisiae* strains with cellulosic bioethanol production as the relevant biotechnology platform.

2. To determine the ethanol production capacity of natural *S. cerevisiae* strains and identify candidate strains for cellulosic ethanol production.
3. To generate hybrid *S. cerevisiae* strains using parental strains with enhanced tolerance
4. To assess the phenotypic characteristics and the ethanol production capacity of the generated hybrid *S. cerevisiae* strains.
5. To investigate the cellular mechanisms involved in temperature tolerance of natural strains.
6. To investigate the cellular mechanisms involved in inhibitor tolerance of natural strains.

1.3 OUTLINE OF THE DISSERTATION

The dissertation is presented in chapter format starting with a review of the relevant scientific literature (Chapter 2), followed by the research conducted to meet the aims of the study (Chapters 3-5). A general discussion and conclusions are presented in Chapter 6.

Saccharomyces cerevisiae is an important eukaryotic model organism that displays genetic and phenotypic plasticity and has been used in a variety of biotechnological and industrial applications. An overview of the diversity of this strain and its biotechnological application with specific reference to the biofuel industry, including the stress responses that allow this organism to persist in various environments, as well as the molecular mechanisms involved in these responses, is presented in Chapter 2.

Natural *S. cerevisiae* strains represent a valuable source of genetic and phenotypic diversity within this species. The first aim with specific objectives 1 and 2, is addressed in Chapter 3 and focuses on the phenotypic characterisation of natural *S. cerevisiae* strains for use as a microbial host in the production of cellulosic bioethanol. Environmental conditions assessed represented conditions typically encountered during the production of cellulosic bioethanol. Several natural *S. cerevisiae* strains were evaluated with regard to their phenotypic characteristics as well as their fermentation performance. Strains with extreme environmental tolerances (inhibitor and temperature) were identified.

It has been demonstrated that hybrid heterosis can be obtained when *S. cerevisiae* strains are subjected to various hybridisation techniques. In Chapter 3, classical genetics are used to determine whether best parent heterosis can be achieved, specifically characteristics important for cellulosic bioethanol production. Aim 2 with specific objectives 3 and 4, is covered in this chapter. Selected strains that displayed unique and/or relevant capabilities were hybridised using spore-to-spore mating to generate genetic diversity by combining different genomes. The progeny were assessed for phenotypic characteristics and fermentation performance, resulting in the isolation of a limited number of strains with enhanced characteristics.

Inhibitor and temperature tolerance represent two important characteristics required for a successful microbial host for cellulosic ethanol production. Chapter 5 therefore aims to investigate the cellular mechanisms of two natural *S. cerevisiae* strains with higher temperature and inhibitor tolerance. This chapter addresses the last aim of the study, specifically objectives 5 and 6. Specific and common cellular mechanisms that allow for inhibitor and temperature tolerances were identified.

Chapter 6 addresses the outcomes of the study and discusses the limitations and future prospects regarding natural *S. cerevisiae* strains as a microbial host for biotechnological applications in general and specifically cellulosic bioethanol production.

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CHAPTER 2

SACCHAROMYCES CEREVISIAE AS A MICROBIAL HOST

CHAPTER 2: *SACCHAROMYCES CEREVISIAE* AS A MICROBIAL HOST

2.1 INTRODUCTION

The unicellular budding yeast *Saccharomyces cerevisiae* is the preferred host for use in industries that require microbial assistance (Steensels et al. 2014). This species has GRAS (generally regarded as safe) status due to its application in the food and alcoholic beverage industry. Furthermore, the ease of cultivation, genetic manipulation and historic long use in the baking, brewing and distillery industries, make it an ideal platform for industrial and biotechnological applications (Mukherjee et al. 2014).

Saccharomyces cerevisiae is the first eukaryotic organism to have its full genome sequenced (Goffeau et al. 1996; Goddard and Greig 2015) and is the model eukaryotic system for use in molecular biology studies. It's genome is the best annotated and most amenable to genetic manipulation and analysis (Cherry et al. 2012). The use of this organism in scientific research has led to major breakthroughs in genetics, genomics, biochemistry and cell biology (Chambers and Pretorius 2010). *Saccharomyces cerevisiae* is also the superior species in terms of tolerance to environmental stress within the *Saccharomyces sensu stricto* group (Warringer et al. 2011).

Energy and food security are major global concerns that are integral to the current drive towards renewable and sustainable bioenergy production. There is a socio-economic need for energy security as global industrialisation and vehicle transport have increased the demand on the earth's limited fossil fuel reserves. However, the energy requirement for household use outweighs the energy requirement for transport in developing countries. In Sub-Saharan Africa (SSA), only 12 % of the population has access to clean cooking compared to the global average of 57 %. The majority of the population depend on traditional solid biomass as the primary energy source for domestic cooking and heating purposes (Mohammed et al. 2013; IEA 2017). Access to an efficient and clean energy supply is crucial to human development in reducing poverty and improving both health and education levels, especially in rural areas (Deichmann et al. 2011; Mohammed et al. 2013).

The main concerns associated with the use of fossil fuels as primary energy source include accessibility, availability and toxicity. Fossil fuel resource depletion is inevitable with oil, natural gas and coal expected to be exhausted in approximately 42, 58 and 117

years, respectively (Guo et al. 2015). In addition, the security of the oil supply is threatened due to political instability in oil-supplying regions (Agarwal 2007; Goldemberg 2007; Favaro et al. 2013; Vohra et al. 2014). Furthermore, the use of fossil fuels has an adverse effect on the environment because of the greenhouse gas emissions that contribute to climate change and global warming. These factors are the driving force behind the need for an alternative renewable energy source.

Biomass provides a unique opportunity for use as an alternative and renewable feedstock for energy production because it is carbon neutral and has a high-energy conversion rate (Hill et al. 2006; Balat and Balat 2009; Eisentraut 2010; Jiang et al. 2010). However, the sustainability of biofuel production depends on both the feedstock and processing technology. Three main types of renewable feedstocks are available, namely sugar, starch and cellulosic biomass. First-generation biofuels are produced from sugar and starch feedstocks derived from edible plant material such as wheat, maize (corn) and sugar cane (Galbe and Zacchi 2002; Sánchez and Cardona 2008; Pimentel et al. 2009). This makes the use of first-generation biofuels controversial due to the direct competition with food production. The use of first-generation biofuels is therefore not ideal, especially in developing countries where food security is a major socio-political concern (Meyer et al. 2008; Van Zyl et al. 2011; Terrapon-Pfaff et al. 2014).

The African continent presents the largest potential in the world for bioenergy production (a quarter of the projected total by 2050), if the necessary advances in agricultural technology are made (Smeets et al. 2007). Second-generation biofuel production will not only negate the food versus fuel debate, but also presents unique opportunities for economic and social advancement (Lynd et al. 2015). The use of cellulosic biomass as feedstock for second-generation energy production is therefore desirable.

The development of cellulosic bioethanol as a renewable energy source addresses several of the sustainable development goals (SDG), established by the United Nations in 2015 as part of Resolution 70/1 of the United Nations General Assembly: “Transforming our World: the 2030 Agenda for Sustainable Development”. These include affordable and clean energy (SDG 6), climate action (SDG 13) and food security (SDG 2, zero hunger). Furthermore, the technology advancement and innovation required for the viable production of second-generation biofuels using agricultural residues as feedstock

addresses SDG 9, industry, innovation and infrastructure and SDG 12, responsible consumption and production.

2.2 *SACCHAROMYCES CEREVISIAE* AS MICROBIAL HOST

Saccharomyces cerevisiae is of fundamental and industrial importance (Walker and Stewart 2016; Walker and Walker 2018). It is the primary model eukaryotic organism used in fundamental research and has been used in various disciplines including science, medicine, agriculture and food industries (Mustacchi et al. 2006; Oliver 2006). Traditionally, *S. cerevisiae* has a primary role in food fermentations (alcoholic beverages, distilled spirits, bakery products and other fermented foods), as feeds and fodder and as a source of single cell protein (SCP) (Johnson and Echavarri-Erasun 2011). Modern applications include the industrial production of enzymes, small molecular weight metabolites, fuel ethanol and heterologous proteins (Johnson and Echavarri-Erasun 2011). The exploitation of *S. cerevisiae* for various applications are summarised in Table 1.

Saccharomyces cerevisiae was among the first organisms to be designated GRAS for application in foods and the production of native and recombinant products, due to its history of safe consumption, and absence of toxin production. It is the first genetically modified organism (GMO) used for recombinant production of food and feed additives and a genetically modified strain of *S. cerevisiae* was one of the first GMOs to be approved for use in the food industry in the United Kingdom. Furthermore, the established methods for genetic manipulation of *S. cerevisiae* makes it the preferred organism for traditional and modern industrial applications (Johnson and Echavarri-Erasun 2011).

The discovery of enzymes is directly associated with *S. cerevisiae* due to the observation that alcoholic fermentation takes place in cell-free yeast extract, with the word enzymes meaning “in yeast”. It is therefore expected that *S. cerevisiae* is an important source of enzymes for traditional and modern industries (Johnson and Echavarri-Erasun 2011).

Table 2-1: Proteins and enzymes produced by *S. cerevisiae* [summarised from Johnson and Echavarri-Erasun 2011].

Category	Product
Native products	
biocatalytic enzymes	pyruvate carboxylase, reductase
feed enzymes	phytases, SCP
industrial enzymes	chymosin, cytochrome P450, invertase
metabolites	adenosine triphosphate, amino acids, coenzyme A, ergosterol, isoprenoids, lactic acid, lactones, vitamins
Heterologous protein products	
pharmaceuticals	α-interferon, β-endorphin, epidermal growth factor, erythropoietin, glucagon, granulocyte macrophage colony stimulating factor, hirudin, HIV-1 envelope glycoprotein gp120, human growth hormone, human serum albumin, insulin, platelet-derived growth factor, prochymosin, urate oxidase
vaccines	diphtheria-tetanus-pertussis, haemophilus influenzae type B, hepatitis A, hepatitis B
Heterologous non-protein products	
disinfectants	camphor, pinene
food colourants	astaxanthin, zeaxanthin
food flavourants	apritone, methyl-benzoate, neroloidol
fragrances	dihydrocoumarin, phenethyl alcohol, terpenoid
pharmaceuticals	alkaloids, artemisinic acid, flavonoids, hydrocortisone, nicotianamine, polyketides, steroids, taxoids

2.2.1 *Saccharomyces cerevisiae* as platform for mammalian biological studies

Advances in biotechnology, including "–omic" platforms, metabolic engineering and systems biology, have generated renewed interest in *S. cerevisiae* as the primary model organism to study fundamental eukaryotic biological processes, including mammalian cell biology (Foury 1997; Steinmetz et al. 2002; Mager and Winderickx 2005; Mustacchi et al. 2006; Oliver 2006). Furthermore, the basic cellular and molecular mechanisms in humans occur in yeasts and 30 % of human genes associated with disease have yeast orthologs, making it an ideal model system to study human disease mechanisms (Foury 1997; Mager and Winderickx 2005; Mustacchi et al. 2006; Suter et al. 2006). Several genetic approaches are used to understand human gene function in *S. cerevisiae,* including gene disruption, gene marking, targeted mutations, gene-dosage effects, and genome-wide allele-specific expression (ASE). In addition, genome-wide analytical approaches have allowed the development of several databases that assist with establishing the yeast-human relationship (Mager and Winderickx 2005). Currently, several *S. cerevisiae* based screening processes are available that were developed by mutation complementation studies in *S. cerevisiae* expressing human proteins (Baetz et al. 2004; Armour and Lum 2005; Perocchi et al. 2007). Moreover, altering the protein of interest allows the study of acute and degenerative diseases (Dunham and Fowler 2014). *Saccharomyces cerevisiae* is also used in the study of cancers and plays a role in the development of anti-cancer therapies (Bjornsti 2002; Putnam et al. 2005; Matuo et al. 2012; Jönsson et al. 2013; Natter and Kohlwein 2013). *Saccharomyces cerevisiae* is therefore the preferred model eukaryotic organism for understanding human physiology, including disease mechanisms and drug therapies.

2.3 *SACCHAROMYCES CEREVISIAE* ROBUSTNESS

The robustness of *S. cerevisiae* is one of the main features that make this organism an ideal host for use in industrial processes. Robustness is described as the ability of biological systems to maintain phenotypic functionality in the presence of temporary and/or permanent, external (environmental) and internal (genetic) fluctuations (Kitano 2004; Krantz et al. 2009). This is achieved by maintaining stability during various fluctuations, or by adapting to external changes by triggering a series of cellular responses (Kitano 2004; Stelling et al. 2004). The response and adaptation are communicated via signal transduction systems that react to fluctuations by producing

regulatory signals in the form of changes in the concentration of activated signal-pathway components (Krantz et al. 2009). Robustness arises when phenotypes are maintained in the presence of perturbations, whereas organisms evolve when perturbations allow new phenotypes to emerge (Aldana et al. 2007). Genetic robustness is thought to be one of the main mechanism responsible for robustness against environmental and genetic fluctuations in single-celled microbial organisms.

2.3.1 Genetic robustness

Living organisms are robust to a variety of genetic changes. Gene regulation networks and metabolic pathways re-organise and rewire to maintain stability and reliability when confronted with point mutations, gene duplications and gene deletions (Aldana et al. 2007). Gene duplication is a major driver of both robustness and evolution where the accumulation of mutations in one copy of the gene allows the parental and duplicated gene to diverge (Diss et al. 2017). Genetic divergence can be divided into four categories. Non-functional genetic divergence occurs when one gene copy is silenced. Neo-functional genetic divergence arises when one gene copy retains the original function whilst the second copy develops a new function. In sub-functional genetic divergence, the two gene copies have complementary functions, which together perform the original function. During dual-functional genetic divergence, the two copies display functional overlap, with each copy able to compensate for loss of function of the paralog (Aldana et al. 2007; Diss et al. 2017). Sub-functional genetic divergence requires the presence of both copies to maintain interaction, thus decreasing robustness, whereas dual-functional genetic divergence contributes to mutational robustness by buffering deleterious mutations (Diss et al. 2017).

The hypothesis that gene duplication is the major driver of robustness remains controversial due to the instability of genetic redundancy (Aldana et al. 2007; Li et al. 2010; Diss et al. 2017). This instability is due to the evolution of duplicated genes where complete redundancy of paralogs is temporary, followed by non-functionalisation that leads to loss of duplicates (Li et al. 2010). To persist, duplicate genes have to diverge through sub-functionalisation and/or neo-functionalisation (Li et al. 2010; Keane et al. 2014).

2.3.1.1 Functional redundancy

Functional redundancy is essential for mutual buffering and is achieved by two main mechanisms. In dual-functionality, gene paralogs share functional similarity where one gene copy compensates for the loss of its paralog (Li et al. 2010). Gene duplication therefore produce isozymes that function during variable environmental and physiological conditions (Maltsev et al. 2005). Alternatively, enzymes performing related functions are distributed on alternate pathways to compensate for an obstruction of a specific pathway that allow for the uninterrupted flow of energy and precursor metabolites through a variety of adaptive mechanisms (Maltsev et al. 2005; Li et al. 2010). This is especially applicable in the central metabolism pathways including glycolysis, the pentose phosphate pathway (PPP) and the tricarboxylic acid cycle (TCA).

Functional redundancy between paralogs is based on similarities in gene expression and genetic interaction. In addition, genes with limited functional overlap, buffer each other through transcriptional reprogramming and differential expression (Li et al. 2010). Functional redundancy is also achieved through biological organisation, including protein folding, homeostasis, metabolic flux and vitality (De Visser et al. 2003). This functional redundancy in cellular components and pathways are obtained through various mechanisms, including repair and protection systems, feedback control, modularity, cellular complexity, metabolic networks and hierarchy (De Visser et al. 2003; Stelling et al. 2004; Stenuit and Agathos 2015).

2.3.2 Environmental robustness

There is a relationship between phenotype mutational robustness (accumulation of hidden genetic variation in paralog genes) and the potential of a population to generate phenotypic variation (Espinosa-Soto et al. 2011). This is supported by the observation that robust systems contain increased neutral mutations. These mutations do not remain neutral, but become detectible, causing phenotypic effects in altered environments (Wagner 2005; Keane et al. 2014). This phenomenon is referred to as phenotypic plasticity, where the same genotype produce different phenotypes depending on environmental influences (Espinosa-Soto et al. 2011). Environmental change therefore triggers phenotypic plasticity in some individuals of a population. If the phenotype is beneficial, genetic assimilation occurs, stabilising the phenotype and rendering it independent from environmental factors.

Robustness is driven by direct adaptive benefits (high mutation rates, large populations and asexual reproduction), efficient sensory systems, functional redundancy of cellular components and optimisation of metabolic and regulatory processes (De Visser et al. 2003; Stelling et al. 2004; Maltsev et al. 2005). Robustness thus requires increased complexity and energy production and biological systems therefore display robustness against a limited number of environmental challenges as the energy cost of adaptation is high (De Visser et al. 2003; Maltsev et al. 2005).

2.4 *SACCHAROMYCES CEREVISIAE* PHYLOGENY

There is a broad correlation between phylogenetic relationships and phenotype diversity (Liti et al. 2009; Schacherer et al. 2009), where specific genomic profiles related to certain phenotypes are associated with specific lineages (Skelly et al. 2013). The population organisation of *S. cerevisiae* consists of distinct geographical and mosaic lineages, which supports the theory that human association contributed to crossbreeding and the generation of new arrangements of established variations (Liti et al. 2009; Schacherer et al. 2009; Peter et al. 2018). This suggests that the relationship between ecological niche and phenotype is due to a common genetic lineage (Warringer et al. 2011).

Five *S. cerevisiae* lineages (European (wine), West African, Sake, North American and Malaysian) that are representative of specific genomic clades, and a mosaic group have been identified (Liti et al. 2009; Strope et al. 2015; Peter et al. 2018). These distinct lineages indicate an association between geography, environment and the level of human interaction (Clowers et al. 2015; Strope et al. 2015; Peter et al. 2018). The Malaysian lineage is distinct from other lineages which may be related to chromosomal rearrangements (Cubillos et al. 2011; Marie-Nelly et al. 2014; Liti 2015).

Natural Chinese isolates display a strong population organisation with twice the extent of genetic diversity identified in *S. cerevisiae* isolates from the rest of the world (Wang et al. 2012). These highly diverged natural Chinese isolates form several distinct clusters that does not group with any of the five lineages. The Taiwanese cluster represents the most divergent natural lineage, indicating that East Asia may represent the geographic origin of *S. cerevisiae* (Liti 2015; Peter et al. 2018). Furthermore, non-Asian strains share a single Chinese origin and do not derive from different Chinese clusters (Peter et al. 2018).

Industrial and natural strains form two separate clades, separated by a large group of mosaic strains within the phylogenetic landscape of *S. cerevisiae* (Peter et al. 2018). Natural Mediterranean oak strains group with industrial strains, whereas sake strains associate with natural clades. Furthermore, some lineages represent characteristics typical of specific fermentation processes (wine, sake, beer and lager fermentations) representing industrial strains (Fay and Benavides 2005; Schacherer et al. 2009). The mosaic clade has recombinant genomes that reflect a mixed ancestry, possibly due to outcrossings between genetically distinct lineages (Liti 2015; Peter et al. 2018). Three distinct groups of mosaic strains that are associated with human-related environments, can be identified, emphasising the role of human association in determining the population organisation of *S. cerevisiae* (Peter et al. 2018). In addition, the majority of clinical isolates aligned with the mosaic group (Strope et al. 2015).

Strains from South Africa are not adequately represented in the current phylogenetic profile of *S. cerevisiae.* South Africa therefore represents a diverse geography and environment, but also has an established wine industry. Thus, the possibility that diverse *S. cerevisiae* strains with unique characteristics may occur, could be exploited for the production of cellulosic bioethanol.

2.5 *SACCHAROMYCES CEREVISIAE* GENETIC DIVERSITY

Saccharomyces cerevisiae species are diverse and strains within this species display a high level of genetic variance (Steensels et al. 2014). Industrial strains are often polyploid or aneuploid with an unstable mating type, whereas laboratory strains are usually haploid with a stable mating type and natural strains are mainly diploid with an unstable mating type (Rainieri et al. 2003; Steensels et al. 2014; Peter et al. 2018). The ploidy of *S. cerevisiae* may be associated with human activity as aneuploidy and polyploidy are mostly observed in industrial strains (Peter et al. 2018). The diploid nature of natural strains may provide these strains with a mitotic growth advantage and thus increased viability, and is also associated with increased genetic diversity (Katz Ezov et al. 2006; Peter et al. 2018).

The whole-genome duplication of *Kluyveromyces waltii* 100 million years ago established the *Saccharomyces* lineage (Kellis et al. 2004), illustrating that changes in the ploidy level have profound genetic and phenotypic effects. An extra copy of a gene, chromosome or

genome promote transcriptional network rewiring and allow duplicated genes the ability to change or mutate and gain new or adapted functions, increasing genetic and phenotypic plasticity (Goddard and Greig 2015; Mattenberger et al. 2017; Peter et al. 2018).

Natural strains have a high degree of genetic variability mainly due to reproduction, which allows for reshuffling of the genome and genetic recombination (Steensels et al. 2014). Gene amplifications, spontaneous mutations, single nucleotide polymorphisms (SNPs), insertions and deletions (indels) and gene copy number variation (CNV) are also observed (Carreto et al. 2011; Bergström et al. 2014; Steensels et al. 2014; Peter et al. 2018). These events allow for genome plasticity, which can alter the characteristics of the generated offspring, leading to an increase in biodiversity (Carreto et al. 2008). Additionally, the CNV observed in natural strains may permit abnormal and/or defective meiosis, increasing the possibility of recombination to occur (Rainieri et al. 2003). Furthermore, the ability of natural *S. cerevisiae* strains to switch mating type along with sporulation allows for genome renewal. Genome renewal subsequently permits elimination of lethal recessive mutations as well as enabling recessive heterozygous mutations to become homozygous, thereby influencing the phenotype (Rainieri et al. 2003; Steensels et al. 2014).

A large portion of the genomic variability displayed by natural *S. cerevisiae* strains are within the subtelomeric regions and the transposable element (Ty-element) insertion sites, suggesting that these are major sources of genetic diversity in natural populations, possibly through homologous recombination (Mieczkowski et al. 2006; Carreto et al. 2008; Demeke et al. 2015; Lopandic 2017). Although, genome instability occurs throughout the genome, it is more frequent in Ty elements due to reciprocal translocations, and at subtelomeric regions due to the high frequency of ectopic recombination (Liti and Louis 2005; Guillamón and Barrio 2017). Furthermore, an increased expression of Ty elements relates to an increase in the expression of stress-responsive genes in certain strains (Carreto et al. 2011). Subtelomeric regions are also involved in structural, gene content and copy number variations. These regions are also high in loss-of-function variants, variable open reading frames (ORFs) and horizontal gene transfer (HGT) segments (Bergström et al. 2014; Peter et al. 2018). In addition, a third of quantitative trait loci (QTLs) for ecological important characteristics are in these

regions (Cubillos et al. 2011), suggesting that subtelomeres play a key role in individual quantitative and qualitative variation and supports the importance of these regions in adaptive evolution in natural populations (Liti and Louis 2005; Cubillos et al. 2011). The genetic differences between laboratory and wild type strains may therefore be linked to subtelomeric instability and retrotransposon activity (Carreto et al. 2008).

Different mechanisms exist that enable yeast to adapt to various environmental conditions. These mechanisms function in an integrated manner to provide tolerance against a multitude of environmental conditions. This indicates that environmental adaptation is a polygenic trait that requires the action and interaction of many genes.

2.6 *SACCHAROMYCES CEREVISIAE* PHENOTYPIC PLASTICITY

One of the major reasons for the popularity of *S. cerevisiae* as industrial host organism is its robustness, which relates to the phenotypic plasticity displayed by this organism (Schacherer et al. 2009). Considerable phenotypic diversity exists among different strains of *S. cerevisiae,* as well as among strains from different environments (Mukherjee et al. 2014). The genetic diversity displayed by *S. cerevisiae* strains allows for the phenotypic diversity observed in this organism. However, a higher degree of phenotypic diversity exists that cannot be exclusively attributed to the observed genetic diversity (Bergström et al. 2014). For instance, within the *Saccharomyces sensu stricto* group, *S. cerevisiae* displays the highest phenotypic diversity despite having the lowest genetic diversity (Rainieri et al. 2003; Warringer et al. 2011; Yadav et al. 2016). This phenotypic diversity may contribute to the ability of the organism to adapt to various niches. *S. cerevisiae* is found in natural habitats associated with fruits, trees, soil, insects, human activity and in facultative infections of immune-compromised individuals (Mortimer 2000; Camarasa et al. 2011). There are significant variation in phenotypes including mRNA and protein levels, metabolism, stress tolerance and sporulation efficiency, which correlate with strain niche and may vary by lineage (Clowers et al. 2015). Ecological niche rather than genetic relationship may therefore be a more suitable indicator of phenotype (Salinas et al. 2016). Environmental conditions unique to a habitat modify strain metabolism and physiology, resulting in adaptation and the development of environment-specific traits (Kvitek et al. 2008; Camarasa et al. 2011). Similar geographical regions display internal ecological barriers that create independent niches. These isolated niches inflict selective pressure, forcing populations to diverge (Clowers et al. 2015). Moreover, human

selection contributes to environment-specific properties of strains during industrial processes (Camarasa et al. 2011). Thus phenotype evolution may be determined by environmental conditions and the interactions between the organism and the environment (Kvitek et al. 2008).

Phenotypic variation between individual strains is polygenic and depends on interactions between genetic factors and the environment, i.e. genotype-by-environment (GxE) interactions, which is due to allelic variations having different effects determined by the specific environment (Mackay et al. 2009; Duveau et al. 2017; Peltier et al. 2018). Genetic polymorphism may contribute to the adaptability of strains to their environment and thus play a role in the development of environment-specific phenotypes (Camarasa et al. 2011). Polymorphisms may affect expression by modifying transcription factor (TF) binding, thereby altering the transcription profile of a specific gene (Chang et al. 2013; Weirauch et al. 2014). Furthermore, allele-specific differences allow for ASE through differences in TF binding (Salinas et al. 2016). This is validated by studies that indicate that accessory genes differ between lineages and represent a large portion of genome diversity between individuals (Bergström et al. 2014). ASE is widespread in natural *S. cerevisiae* strains and is responsible for variation in gene expression (Fay et al. 2004; Cubillos 2016). Differentially expressed genes (DEGs) allow for metabolic flux and network regulation and play a major role in the adaptation to environmental conditions and thus phenotypic plasticity (Promislow 2005). Thus, phenotype variation may be due to gene expression variation driven by polymorphisms within coding and non-coding regions (Majewski and Pastinen 2011; Bergström et al. 2014; Salinas et al. 2016). Modification of gene expression patterns promote regulatory elasticity and allow organisms to withstand unfavourable environmental conditions (Cubillos 2016). Alternative association signals, including those involved in gene expression variation, have been indicated as important mechanisms underlying natural phenotypic variation between individuals (Wray 2007; Gerke et al. 2009; Fraser et al. 2012; Parts 2014).

Trait variations may also be due to promoter, terminator, missense and gene duplication mutations (Warringer et al. 2011). Moreover, several variants responsible for polygenic traits modify protein structure, implicating protein-coding variants in polymorphisms (Kumar et al. 2009; Wei et al. 2011; Cubillos et al. 2013). Additionally, certain forms of

non-genetic variation, including prions, might be important factors of phenotypic variation (Halfmann et al. 2012; Liebman and Chernoff 2012; Jarosz et al. 2014).

Genotype-phenotype associations therefore include ploidy, genome content, CNV, SNPs, transcriptional regulation and structural variation (Liti 2015; Salinas et al. 2016; Peter et al. 2018). Phenotypic plasticity depends on the inherent genetic diversity that exists within a single population, including mutations that occur in a miniscule subset of the population. GxE interactions are selected for and thus both the genetic diversity and the environment allow for phenotypic plasticity (Yadav et al. 2016).

2.7 NATURAL *SACCHAROMYCES CEREVISIAE* STRAINS

Industrial (strains used as inoculum in industrial processes), laboratory (strains manipulated for laboratory applications) and natural (strains obtained from natural habitats) *S. cerevisiae* strains differ from each other in genetic and phenotypic properties. Laboratory strains differ from industrial strains in their phenotypic responses to environmental changes, physical changes and nutrient limitations (Gibson et al. 2007). Industrial strains express phenotypes important in industrial processes, including rapid growth, increased production rates and enhanced tolerance (Gibson et al. 2007). Industrial *S. cerevisiae* strains have adapted to artificial environments through increased genetic diversity and phenotypic plasticity (Peter et al. 2018). These environments promote ORF acquisition through HGT, amplification through duplication, and/or loss of genes, leading to variation in genome content and copy number (Peter et al. 2018).

Natural *S. cerevisiae* isolates are genetically and phenotypically distinct, which could be attributed to the diverse ecological niches the organism occupies (Liti et al. 2009; Clowers et al. 2015). These strains display SNPs that may generate rare alleles that are associated with polymorphisms that bring about phenotype variation (Peter et al. 2018). Additionally, an increased number of CNVs are observed in these strains (Bergström et al. 2014; Peter et al. 2018). Moreover, non-annotated ORFs and ORFs acquired from HGT may play a role in modulating the phenotypic landscape of natural *S. cerevisiae* strains (Peter et al. 2018). The genetic variability in *S. cerevisiae* natural strains may also result from outcrossing (Naumova et al. 2005; Liti et al. 2009; Strope et al. 2015). These observations substantiate the idea that phenotypic variation is related to environmental conditions (Bergström et al. 2014; Peter et al. 2018).

Several natural strains display an increased ethanol tolerance in comparison to laboratory and industrial strains (Lewis et al. 2010). In addition, natural *S. cerevisiae* strains have a higher ethanol productivity with increased growth both in the absence and presence of inhibitory compounds, compared to laboratory and industrial strains (Mukherjee et al. 2014; Steensels et al. 2014; Dubey et al. 2016). When comparing the genome of laboratory strains to multi-tolerant natural strains, several SNPs were identified (Wohlbach et al. 2014). In addition, the transcriptome of the natural strains were enriched in functional categories that play a role in stress tolerance and stress-responsive signalling factors (Wohlbach et al. 2014).

Several industries that rely on *S. cerevisiae* as microbial host, including the wine and biofuel industries, use indigenous strains (natural strains associated with a specific environment) that have evolved and adapted to the industrial environment (Steensels et al. 2014). These strains often outperform the industrial strains used in the production of first-generation biofuels (Da Silva-Filho et al. 2005; Basso et al. 2008; Jin et al. 2013; Ramos et al. 2013; Mukherjee et al. 2014; Pereira et al. 2014; Wimalasena et al. 2014; Ruyters et al. 2015). Moreover, natural and industrial strains display similar tolerances to harsh environmental conditions with some natural strains outperforming the industrial strains (Mukherjee et al. 2014). The geographical location and the specific industrial process prescribe the genetic and phenotypic characteristics of the organism (Zhu et al. 2016). This indicates that the diversity of natural strains can be utilised to obtain robust and productive industrial host strains for current and future biotechnological applications.

2.8 SECOND-GENERATION BIOETHANOL

Cellulosic biomass is derived from residual plant material and represents the most abundant feedstock and in addition, it does not compete with food sources. A study conducted in 2007 determined that the global capacity of bioenergy production from forestry and agricultural residues and cellulosic waste streams could reach 76–96 EJ/yr by 2050 (Smeets et al. 2007). Furthermore, the global bioenergy potential of surplus agricultural land could reach 215–1272 EJ/yr with SSA producing 31–317 EJ/yr (Smeets et al. 2007; Nanda et al. 2016). The use of residues provide an alternative energy source that is sustainable, thus reducing environmental pollution (Hahn-Hägerdal et al. 2006; Meyer et al. 2008; Van Zyl et al. 2011; Ho et al. 2014).

The main obstacle for the implementation of cellulosic biofuel production is the high risk of the investment (technological and political) combined with low potential economic returns. Therefore, current technology for cellulosic biofuel production requires significant improvements to make the process efficient and cost-effective (Van Zyl et al. 2011; Lynd et al. 2017). For a bioenergy platform to be effective, sustainability in terms of the technology, economics, society and the environment is required (Bhattacharyya 2012).

2.9 BIOMASS FEEDSTOCK

Biomass consists of two main components, namely high and low molecular weight components (Fig. 2.1). Polysaccharides from the high molecular weight components are the main source for the production of cellulosic biofuels. In addition, the lignin and low molecular weight components can be used to produce high-value products, providing an additional revenue stream. The composition of lignocellulosic biomass depends on the specific feedstock and includes complex components such as lignin, pectin, hemicellulose and cellulose, as well as primary constituents such as sugars and alcohols (Fig. 2.2) (Isikgor and Becer 2015).

An integrated biorefinery approach using both components will increase the economic feasibility of cellulosic biofuel production. To exploit the full potential of biomass, natural, agricultural, industrial as well as municipal cellulosic biomass should also be considered for bioethanol production (Fig. 2.3) (Zabed et al. 2016).

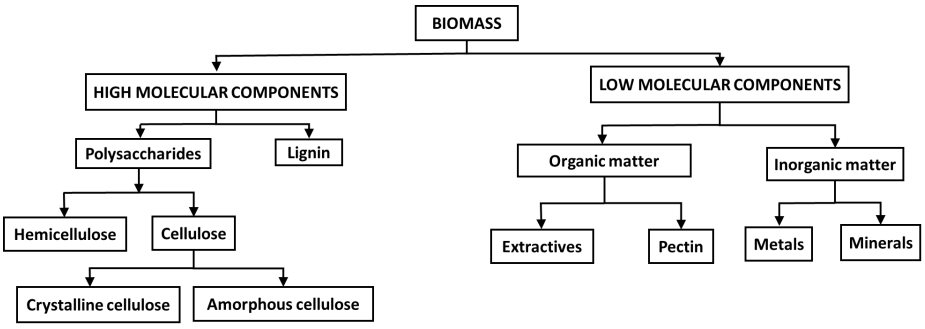

Figure 2.1: The major constituents of biomass are high and low molecular weight components. Low molecular weight components include both organic and inorganic matter that can be recovered. High molecular weight components are the main source of cellulose that can be used as feedstock for bioethanol production [adapted from Nanda et al. 2016].

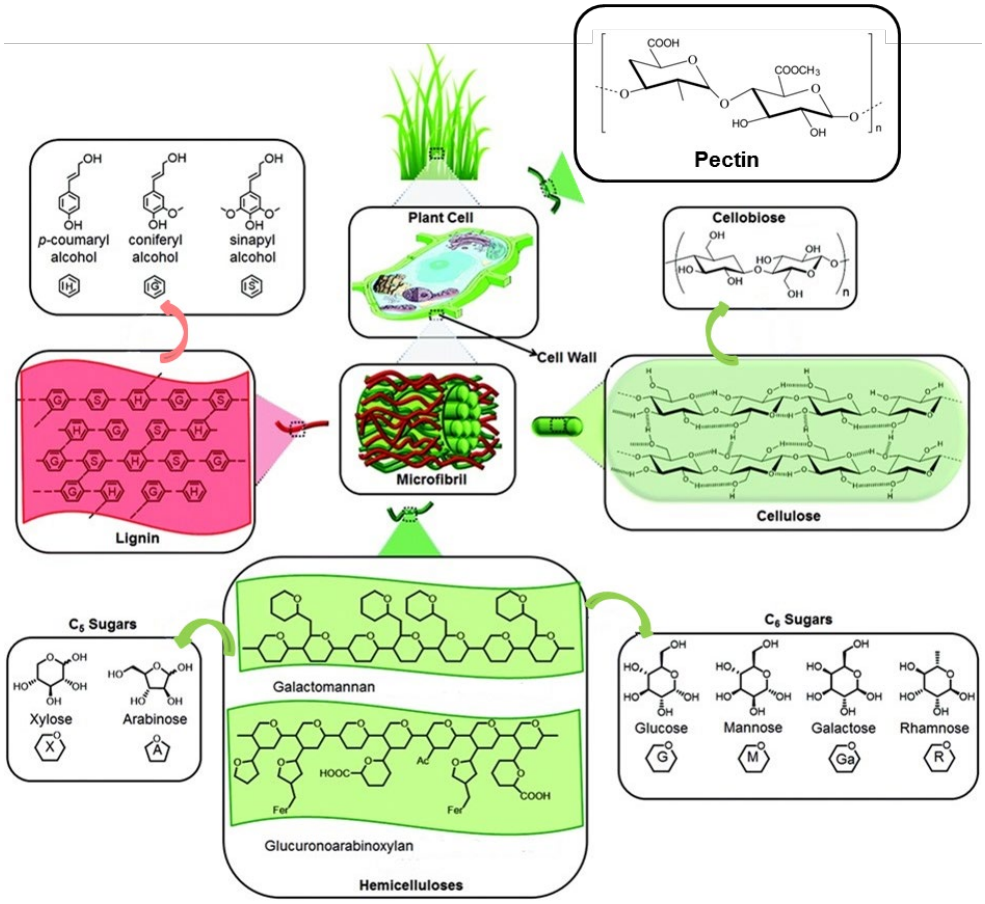

Figure 2.2: The composition of lignocellulosic biomass indicating the complex components (lignin, pectin, hemicellulose and cellulose) and primary constituents (sugars and alcohols) as well as their chemical structure [adapted from Isikgor and Becer 2015].

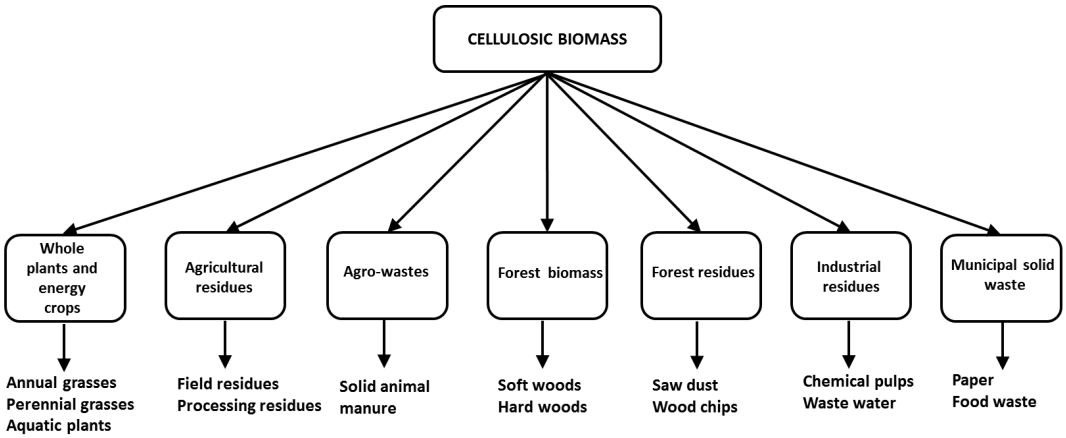

Figure 2.3: Sources and examples of cellulosic biomass. Apart from dedicated plant sources and plant residues, cellulosic material generated during industrial processes can be used as feedstock for bioethanol production [adapted from Zabed et al. 2016].

2.10 CELLULOSIC BIOETHANOL

Two main routes can be used to generate biofuel from biomass, namely thermochemical and biochemical conversion (Fig. 2.4). The production of cellulosic bioethanol via the biochemical route requires three steps, including cellulosic feedstock pretreatment, hydrolysis of the cellulose and hemicellulose into fermentable sugars and the conversion of the sugars into ethanol (Fig. 2.5). Several technologies are available to achieve each of these individual processes, each with their own advantages and disadvantages.

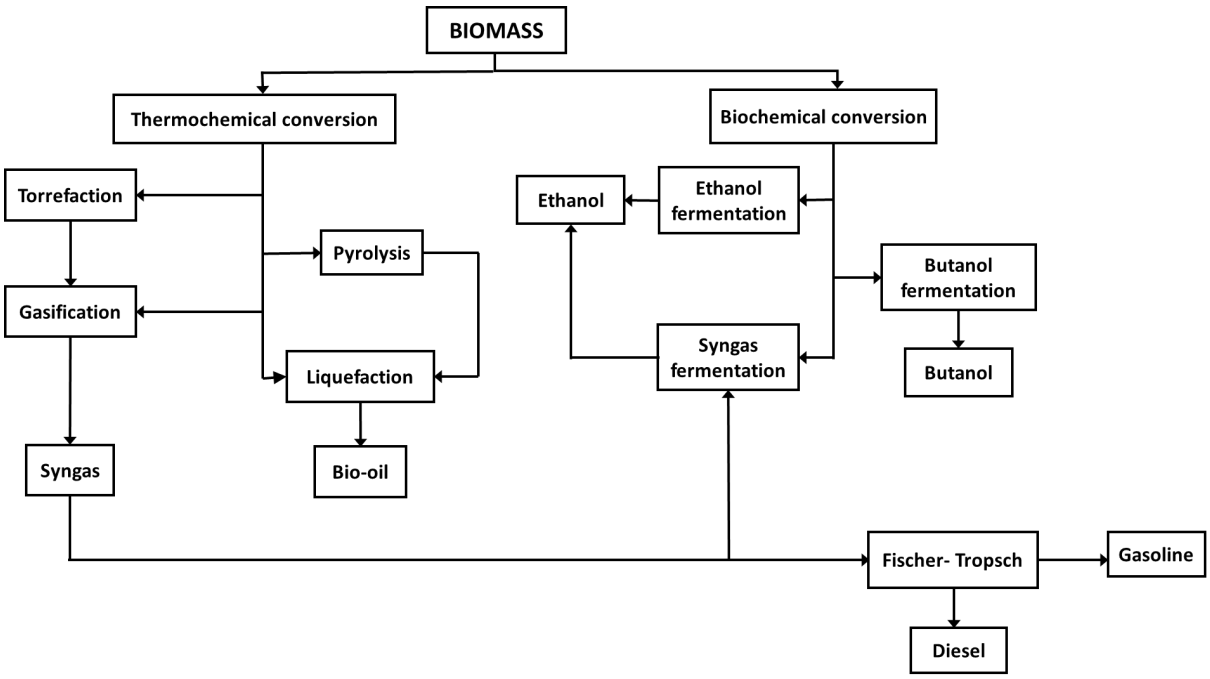

Figure 2.2: Thermochemical and biochemical conversion of lignocellulosic biomass. Several individual processes are required for the conversion of LCB into various liquid fuels including ethanol, butanol, diesel, bio-oil, and syngas. The processes are integrated and diverge and converge at various intervals depending on the specific technology employed [adapted from Nanda et al. 2016].

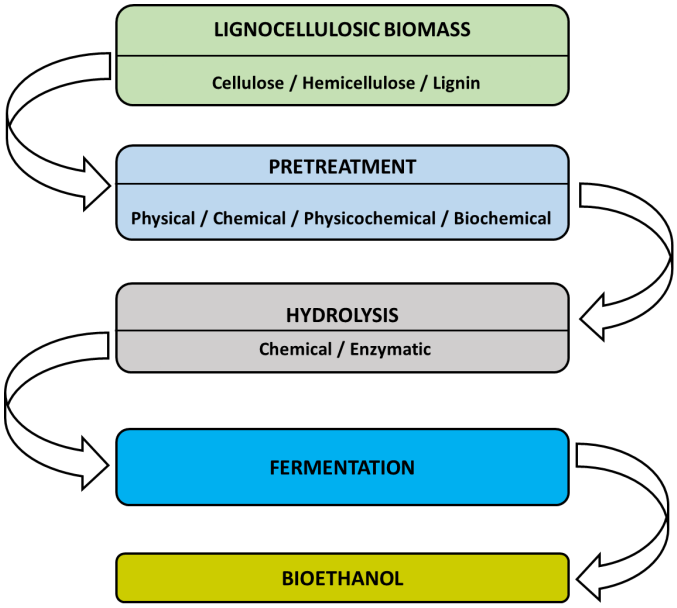

Figure 2.3: Overview of the major steps required for the biochemical production of cellulosic bioethanol. LCB is pretreated to access the cellulose, whereafter the cellulose is hydrolysed into monomeric sugars that are fermented to ethanol.

2.10.1 Pretreatment

The recalcitrant nature of cellulosic material requires pretreatment to increase the accessibility of monomeric sugars for fermentation. Pretreatment methods are divided into four major groups, biological, physical, chemical and physicochemical (Fig. 2.6).

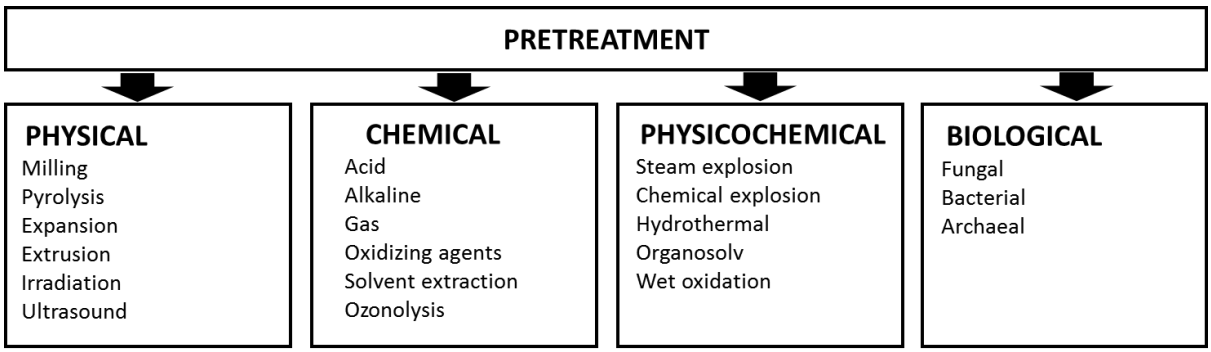

Figure 2.4: The four major pretreatment methods for cellulosic biomass, including the various types of individual processes within each method [adapted from Kumar and Sharma 2017]

Physical pretreatment increases the accessible surface area and pore volume, decreases cellulose crystallinity and allows partial depolymerisation of lignin (Szczodrak and Fiedurek 1996; Rao et al. 2015). Physical pretreatment often requires the use of harsh environmental conditions (extreme temperatures, pH and pressure) to render the cellulosic material accessible for hydrolysis. Furthermore, these methods require additional infrastructure, increasing the capital cost of the process (Brodeur et al. 2011).

Chemical pretreatment allows delignification and decreases the level of polymerisation and crystallinity of cellulose (Szczodrak and Fiedurek 1996; Swain and Krishnan 2015). The compounds (acid, alkali, salt, organic and inorganic solvents and ionic liquids) used in this process disrupt the association of lignin with cellulose, reduce cellulose crystallinity and solubilise the hemicellulose, making the cellulose accessible to enzymatic digestion (Mosier et al. 2005). These compounds are often toxic and/or inhibitory to the fermentation process and affect the osmolarity and pH of the industrial process.

Physicochemical pretreatment increases the surface area of the biomass, decreases cellulose crystallinity and removes hemicelluloses and lignin (Mosier et al. 2005; Alvira et al. 2010). The extreme environmental conditions and the addition of chemical compounds increase the negative effects associated with inhibitory compounds, extreme osmolarity and pH.

Biological pretreatment uses microorganisms (white-rot, brown-rot and soft-rot fungi) to modify the lignin and cellulose structure, separating it from the lignocellulosic matrix (Sarkar et al. 2012). These fungi secrete lignin-degrading enzymes that degrade lignin. Brown-rot fungi affect only cellulose, whereas white- and soft-rot fungi degrade cellulose and lignin (Prasad et al. 2007). Enzymatic pretreatment also releases inhibitory compounds, the nature of which depends on the specific feedstock as well as the microbial host used.

Pretreatment methods depend on the source of the biomass and lead to the formation of various degradation products depending on the biomass type and the specific pretreatment method. These degradation products are often inhibitory to the fermentation and industrial processes. Although pretreatment is the single most

expensive step in the process, it remains essential for the production of cellulosic bioethanol.

2.10.2 Hydrolysis

Hydrolysis completely depends on the pretreatment of cellulosic biomass to yield two fractions, the water-insoluble fraction composed of cellulose and lignin, and the liquid fraction containing hemicellulose. Depending on the pretreatment method, the hemicellulose can be completely hydrolysed into monomeric sugars or is converted to oligosaccharides that require further hydrolysis to obtain monomeric sugars that are fermented to ethanol (Zabed et al. 2016).

Hydrolysis can be enzymatic or chemical. In general, chemical hydrolysis uses two approaches, namely dilute acid treatments at high temperature and pressure with a short reaction time, or concentrated acid treatments at low temperature (Chandel et al. 2007; Balat 2011). Chemical hydrolysis requires recovery or neutralisation of the acids, produces large amounts of waste and is corrosive to equipment. It also causes degradation of the fermentable sugars, leading to the formation of toxic compounds and lowering ethanol production (Zabed et al. 2016). In addition, the chemicals and the specific environmental conditions employed may have inhibitory effects on the downstream fermentation process.

Enzymatic hydrolysis of cellulosic biomass is more precise due to the substrate specificity of the enzymes and reduced inhibitor generation. The synergistic action of many different enzyme activities are required, but, hydrolysis is often incomplete with lower than expected theoretical yields (Zabed et al. 2016). Enzymatic hydrolysis is achieved in two ways: commercial cellulolytic enzymes are added to the pretreated cellulose or cellulolytic microbial organisms are used to hydrolyse the pretreated cellulose (Zabed et al. 2016). Both scenarios have received scientific attention with reasonable success. As with chemical hydrolysis, enzymatic and microbial hydrolysis require specific environmental conditions (pH, temperature, pressure) for optimal performance that may not correlate with the industrial process. This depends on both the organism and/or enzymatic cocktail, as well as the specific feedstock.

2.10.3 Fermentation

Pretreatment and hydrolysis generate a variety of sugars of which the monomeric sugars can be microbially converted to bioethanol. Ethanol fermentation can be either submerged or solid state. Submerged fermentation requires mixing of the solid hydrolysed material with water to make a fermentation mash, whereas only the surface of the biomass is saturated with water (in solid-state fermentation) (Zabed et al. 2016). Submerged fermentation can be batch, fed-batch or continuous, depending on the kinetic properties of the organism and the feedstock type. Batch culture is the easiest method and is performed in a closed culture system, where the fermenting microorganism is inoculated into defined fermentation media and allowed to ferment until the nutrients are depleted (Balat 2011). In fed-batch fermentation systems, microorganisms function at low substrate concentrations with an increasing ethanol concentration during the course of the fermentation process. This system is often used for commercial ethanol production. In continuous fermentation, feed containing substrate, culture medium and nutrients is pumped continuously into the fermentation vessel during the fermentation process (Balat 2011).

Depending on the biomass, type and concentration of sugars in the hydrolysates, microbial host and process conditions of the fermentation, the ethanol yield differs considerably (Zabed et al. 2016). As with the previous processes, fermentation is also associated with inhibitory compounds and conditions, of which ethanol accumulation is the most problematic.

2.11 BIOETHANOL PRODUCTION TECHNOLOGIES

The cost of bioethanol production can be reduced by integrating the various steps required during production (Parisutham et al. 2014). Enzymatic hydrolysis of cellulose and fermentation of the monomeric sugars can be combined in various different processes (Fig. 2.7). These include separate hydrolysis and fermentation (SHF) (Hamelinck et al. 2005), separate hydrolysis and co-fermentation (SHCF) (Gírio et al. 2010), simultaneous saccharification and fermentation (SSF) (Kádár et al. 2004), and simultaneous saccharification and co-fermentation (SSCF) (Teixeira et al. 1999). Simultaneous saccharification, filtration and fermentation (SSFF) is a hybrid technique that combines SHF and SSF (Ishola et al. 2013). During consolidated bioprocessing (CBP), an alternative to SSF/SSCF, hydrolysis and fermentation of both types of soluble sugars

as well as the production of hydrolysing enzymes for biomass conversion into ethanol, are integrated into a single step by using microbial organisms to perform both hydrolysis and fermentation (Parisutham et al. 2014).

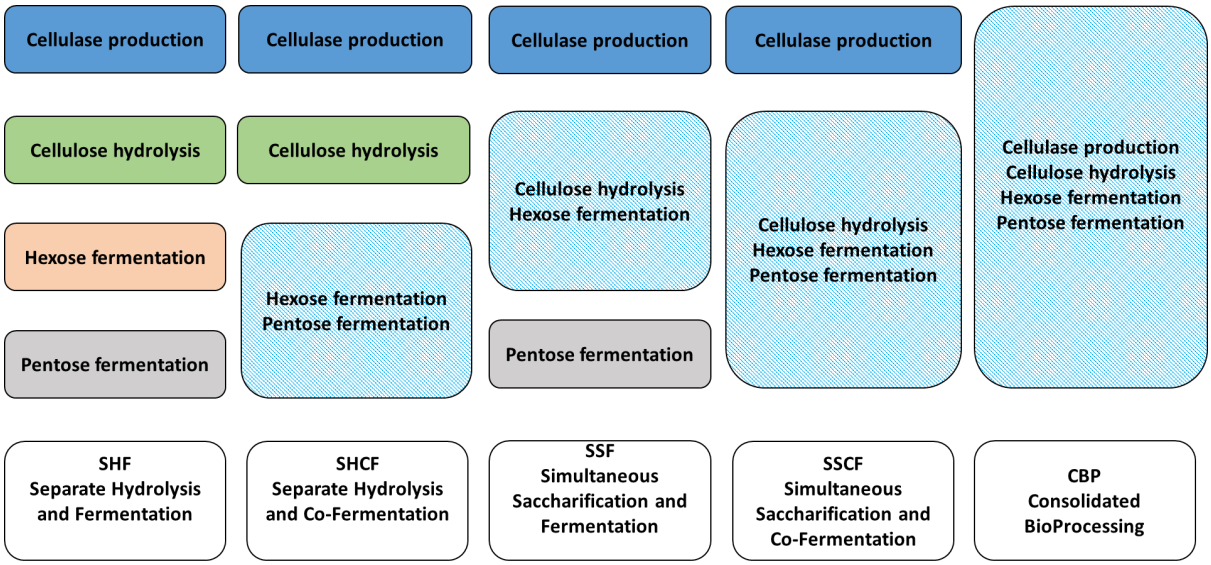

Figure 2.5: Overview of existing and proposed bioethanol production technologies. The various steps required for bioethanol production are integrated in different combinations to produce technologies that can potentially be used to decrease operational costs.

CBP is considered one of the possible technologies to reduce cost when producing cellulosic bioethanol (Lynd et al. 2005). However, microbial organisms able to hydrolyse cellulose are not the most effective at fermenting the released sugars, conversely the most efficient alcoholic fermentative organisms are not cellulolytic (Bothast et al. 1999; Alfenore et al. 2003). Thus, two scenarios are possible to achieve CBP: effective fermentation ability can be genetically engineered into cellulolytic organisms or cellulolytic capabilities can be engineered into efficient fermentative organisms (Lynd et al. 2002).

2.12 CELLULOSIC INHIBITOR TOXICITY

Depending on the biomass source (Fig. 2.2), various inhibitory and/or toxic compounds are introduced into the industrial process during the cellulosic bioethanol production process. These include chemicals, minerals and salts used in the specific industrial process, as well as organic and inorganic matter derived from the various residual waste

streams. In addition, the cellulosic feedstock composition depends on the specific raw material, and hydrolysis of this material is associated with inhibitory compounds (Fig. 2.8). Aside from the inhibitory compounds that are introduced into the industrial process, inhibitory and/or toxic compounds are also produced during all three steps required for bioethanol production. Pretreatment produces inhibitory compounds during the degradation of the raw materials, hydrolysis of the feedstock releases inhibitory compounds and the fermentation process generates inhibitory compounds. These compounds can be inhibitory to both the fermentation process as well as the fermenting organism.

The pretreatment process is responsible for the majority of inhibitory compounds present during cellulosic bioethanol production. The pretreatment method needed to render the biomass accessible for hydrolysis requires the addition of catalysts such as acids, alkalis, ionic and organic solvents that can be toxic and/or inhibitory during hydrolysis and fermentation (Fig. 2.6) (Klinke et al. 2004; Kumar and Sharma 2017). In addition, the pretreatment conditions (temperature, pressure, pH and redox conditions) exacerbate the toxicity of the hydrolysate (Klinke et al. 2004). Metals are also released from the equipment and the recirculation of process water results in high salt concentrations.

The most common inhibitory compounds released from the degradation of the cellulosic components during the pretreatment process can be divided into three groups. These include aldehydes (furans and aliphatic), weak organic acids (aliphatic and uronic) and phenolic and aromatic compounds (Fig. 2.8) (Klinke et al. 2004; Caspeta et al. 2015; Petrovič 2015).

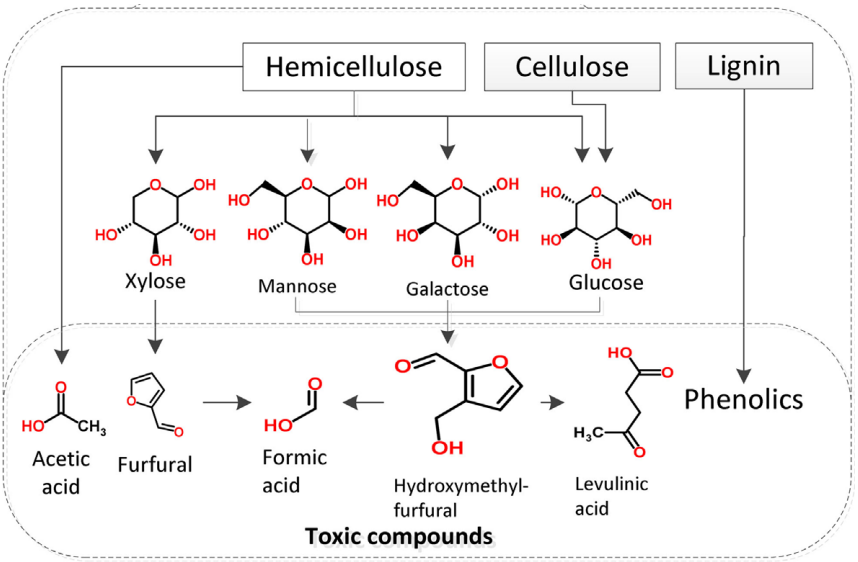

Figure 2.8: Inhibitory compounds released during the degradation of cellulosic biomass. Degradation products released from the main constituents include acetic acid and phenolic compounds released from hemicellulose and lignin. The breakdown of the monomeric sugars releases weak acids and phenols [adapted from Caspeta et al. 2015].

Furan aldehydes most commonly found in cellulosic hydrolysate include 5- hydroxymethylfurfural (HMF) and furfural and are due to the dehydration of pentose and hexose sugars, respectively. The degradation of these furan aldehydes in turn leads to the production of weak acids, including formic and levulinic acid (Palmqvist and Hahn-Hägerdal 2000a; Almeida et al. 2007). Acetic acid is generated when the acetyl groups from the xylan backbone of the hemicellulose fraction are cleaved (Chen et al. 2012). In addition, phenolic and aromatic compounds (4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, coniferyl aldehyde, dihydroconiferyl alcohol, syringaldehyde, syringic acid and vanillin), as well as heavy metal contaminants, are released during the partial breakdown of lignin (Christopher et al. 2014; Jönsson and Martín 2016).

The specific toxic compounds and concentration of these compounds present in cellulosic hydrolysate are dependent on the pretreatment method and the type of feedstock (Klinke et al. 2004; Petrovič 2015). These inhibitory compounds have a negative effect on cell viability that leads to a reduction in fermentation efficiency, thus decreasing the feasibility of the process (Larsson et al. 1999a; Larsson et al. 2000; Mukherjee et al. 2014).

2.12.1 Effect of inhibitor toxicity on microbial fermentation

The compounds generated after biomass degradation exhibit cytotoxicity and inhibits microbial growth, metabolism and ethanol yield (Klinke et al. 2004; Van Maris et al. 2006; Almeida et al. 2007; Mills et al. 2009; Pienkos and Zhang 2009). These compounds display a structure-activity relationship (SAR) that depends on the microbial strain and is ascribed to differences in cell membranes and metabolism (Xie et al. 2016). The inhibitory profiles of aromatic compounds indicate that aldehydes have the highest toxicity, followed by acids, with alcohols having the lowest toxicity (Klinke et al. 2004; Zaldivar & Ingram 1999; Zaldivar et al. 1999; Zaldivar et al. 2000; Jönsson et al. 2013; Adeboye et al. 2014; Ando et al. 1986). In *S. cerevisiae,* furan aldehydes and phenols inhibit growth and ethanol production rate, but not ethanol yield, with phenols having the highest inhibitory effect on fermentation (Ranatunga et al. 1997; Larsson et al. 2000). Low molecular weight organic and phenolic compounds have a higher inhibition effect due to their ability to penetrate cell membranes, whereas high molecular weight compounds influence the activity and expression of sugar and ion transporters in the cell membrane (Klinke et al. 2004; Xie et al. 2016). Furthermore, the SAR also correlates with the hydrophobicity of the compounds (Ando et al. 1986; Zaldivar et al. 1999; Zaldivar and Ingram 1999; Zaldivar et al. 2000; Jönsson et al. 2013; Adeboye et al. 2014) with an increase in the methoxy groups of phenolic compounds corresponding with high hydrophobicity and toxicity (Klinke et al. 2004). Phenolic compounds are insoluble in aqueous solutions and can be incorporated into cellular membranes (Heipieper et al. 1994) where they interfere with protein function and elicit changes in the protein to lipid ratio (Xie et al. 2016). This affects cellular functions such as sorting and signalling and cause membrane swelling (Caspeta et al. 2015).

Furan aldehydes damage DNA, reduce translation and inactivate enzymes (Modig et al. 2002; Allen et al. 2010; Ask et al. 2013a). Yeast cells use oxidoreductases to metabolise furfural and HMF to less toxic alcohols (Ask et al. 2013a; Zhao et al. 2015). This conversion requires NAD(P)H as a cofactor, reducing the redox cofactor concentration and increasing the energy requirement of the organism (Taherzadeh et al. 1999; Sárvári Horváth et al. 2003; Ask et al. 2013a). Ethanol fermentation in the presence of furfural thus reduces glycerol and increases acetate production to balance the redox potential due to the increased demand for cofactors (Palmqvist et al. 1999a; Sárvári Horváth et al. 2003; Ask et al. 2013b). *Saccharomyces cerevisiae* is more tolerant to HMF than furfural

(Taherzadeh et al. 1999) because of the lower permeability and conversion efficiency of HMF (Larsson et al. 1999a). Accumulation of reactive oxygen species (ROS) induced by furfural damages the mitochondria and vacuoles, resulting in a reduction of glucose consumption rates, and increases the oxidative stress as these organelles play a role in maintaining the redox balance (Allen et al. 2010).

Acid toxicity depends on the membrane permeability, toxicity of the anionic forms of the acid and pH (Palmqvist and Hahn-Hägerdal 2000a; Van Maris et al. 2004). At a low pH, weak acids are in the undissociated form and are able to permeate the cell membrane. Inside the cell, the undissociated acids dissociate due to the intracellular pH, leading to intracellular acidification (Ullah et al. 2012). This lowers the intracellular pH and excess protons are pumped out of the cells using several mechanisms, including proton translocation via the plasma membrane H^+-ATPase, mediated by ATP hydrolysis (Holyoak et al. 1996; Van Maris et al. 2004). This process decreases intracellular ATP levels because ATP is utilised to maintain the internal pH, with a concomitant reduction in biomass yield (Viegas and Sá-Correia 1991; Verduyn et al. 1992; Holyoak et al. 1996). The anions accumulated inside the cells may reach toxic concentrations, which impair essential metabolic functions (Krebs et al. 1983), including a reduction in enolase and phosphoglyceromutase activity, thereby affecting glycolysis (Pampulha and Loureiro-Dias 2000). Furthermore, weak acids accumulate in the cytoplasmic membrane and increase membrane permeability and fluidity, reduce energy transduction and interfere with membrane protein function (Dunlop 2011). An increase in membrane permeability allows the release of cellular components and is detrimental to energy maintenance in the cell as it leads to a decrease in ATP levels, reduced ATP synthesis and a reduced proton motive force (Dunlop 2011).

Aromatic and carboxylic acids are usually not inhibitory, whereas phenols and phenol derivatives (phenol aldehydes and phenol ketones) are strong inhibitors (Klinke et al. 2004) with 4-hydroxy-3-methoxycinnamaldehyde being the most toxic phenolic compound (Adeboye et al. 2014). The effect of these inhibitory compounds is concentration dependent: phenols and furan aldehydes are inhibitory at concentrations of 1 $g.L^{-1}$, while organic acids only display an effect at concentrations higher than 2 $g.L^{-1}$ (Caspeta et al. 2015). In contrast phenylpropane unsaturated acids (4-hydroxycinnamic

acid and ferulic acid) severely inhibit productivity at low concentrations (1.4 mM – 10 mM) (Klinke et al. 2004; Adeboye et al. 2014).

The effect of inhibitory compounds are enhanced when in the presence of other compounds and can be additive or synergistic (Palmqvist et al. 1999b; Zaldivar et al. 1999; Zaldivar et al. 2000; Klinke et al. 2004). Synergistic inhibition has multiple effects on cell growth, membrane integrity and metabolite biosynthesis caused by insufficient and inconsistent repair mechanisms (Ding et al. 2011). Acetic acid and furfural act synergistically in decreasing the specific growth rate (Palmqvist et al. 1999b), suggesting an increased energy demand to excrete acid anions, protons and furfural out of the cell, as well as the ROS produced during furfural assimilation. However, certain compounds are advantageous to microbial performance. In general, sub-inhibitory levels of phenols, acetic acid and furfural improve ethanol yields of both glucose and xylose-fermenting microorganisms (Taherzadeh et al. 1997; Palmqvist et al. 1999b; Klinke et al. 2004). For example, the addition of acetaldehyde alleviates inhibition of acid-pretreated birch hemicellulose hydrolysates in *S. cerevisiae,* requiring no detoxification step (Barber et al. 2000).

In this study a synthetic cocktail inhibitory cocktail was used that consisted of compounds generally contained in pretreated lignocellulosic biomass. The cocktail was composed of two aliphatic acids (formic- and acetic acid), two furaldehydes (furfural and HMF) and two aromatic compounds (cinnamic acid and coniferyl aldehyde) (Martín and Jönsson 2003). A 100 % inhibitor cocktail represents the concentrations at which the respective compounds are completely inhibitory to *S. cerevisiae* (Larsson et al. 1999a; Larsson et al. 2000).

The influence of the inhibitory compounds can be minimised through the removal of these compounds by detoxification. Chemical detoxification methods include water washing, overliming, vaporisation and ion exchange absorption (Larsson et al. 1999b; Palmqvist and Hahn-Hägerdal 2000b). However, these methods have disadvantages including the use of freshwater, generation of wastewater, loss of cellulose particles and sugars, incomplete removal of inhibitors and lower ethanol yield (Mussatto and Roberto 2004; Jönsson et al. 2013). Biodetoxification circumvents the drawbacks associated with chemical detoxification as it relies on microorganisms to degrade the toxins by secreting peroxidase or laccase enzymes (López et al. 2004; Nichols et al. 2005; Yu and Stahl 2008;

Wierckx et al. 2010; Zhang et al. 2010). For instance, a genetically engineered *S. cerevisiae* strain expressing laccase is able to detoxify phenols, increasing the ethanol yield (Larsson et al. 2001). Additional detoxification steps increase the production cost and time, further limiting the economic feasibility of cellulosic bioethanol production (Von Sivers and Zacchi 1996; Palmqvist and Hahn-Hägerdal 2000a).

Aside from the toxins produced during the three steps required for bioethanol production, the high osmolarity of the hydrolysate, the elevated temperature of the fermentation process, pH range, redox conditions and the ethanol produced during the fermentation process, further exacerbate the toxic nature of cellulosic fermentation. This results in reduced growth and ethanol yield (Liu 2011). The microbial host for the production of cellulosic bioethanol thus requires unique characteristics such as osmotolerance, ethanol tolerance, tolerance to toxic compounds and thermotolerance (Mukherjee et al. 2014).

2.13 TEMPERATURE AND PRODUCTION OF CELLULOSIC BIOETHANOL

Fermentative ethanol production generates heat through the metabolic activities of the host organism, increasing the operating temperature (Abdel-Banat et al. 2010; Kumar et al. 2013). This increase in temperature affects the productivity and viability of the microbial host, leading to a decrease in product formation. The production of heat is exacerbated in regions where geographical temperatures are high and cooling of fermentation systems is required to maintain the processes at the desired temperature (Kumar et al. 2013). Maintaining the fermentation process at an optimum temperature increases production cost as additional infrastructure and processes are required. Higher operational temperatures are, however, preferred when producing cellulosic bioethanol as the optimal temperature for lignocellulose hydrolytic enzymes are between 45–50 °C (Grajek 1986; Lu et al. 2012). Temperature-tolerant host organisms will be able to maintain cell viability, have enhanced metabolic activity and therefore increased productivity at higher temperatures (Yang et al. 2013). This would allow the use of an increased operational temperature that is compatible with optimal cellulase and hemicellulase activity. In addition, the degree of cooling required is decreased, reducing operational costs (Yang et al. 2013; Saini et al. 2015) and the water requirements, thereby enhancing the sustainability of the process.

2.13.1 Effect of temperature on microbial fermentation

Temperature plays an important role during fermentation processes and affects both the kinetics of the process and the viability of the fermentative organism. An increase in temperature has damaging effects on cellular components including biomolecules (proteins, DNA and RNA) and membranes (cytoplasmic, mitochondrial, endoplasmic reticulum (ER) and vacuole) (Goldberg 2003; Woo et al. 2014). In addition, carbon metabolism is reprogrammed, growth is inhibited and cell death may occur (Goldberg 2003; Morano et al. 2012).

The energy demand of a cell increases as the temperature increases, which leads to an increase in glucose uptake, with a concomitant increase in glycerol, ethanol and acetic acid production. Carbon flux into the TCA cycle is also enhanced to meet the energy demand, further increasing acetic acid production (Woo et al. 2014). The increased acetic acid production has adverse effects on the cell, leading to an increase in the production of ROS. The ROS production rate also increases during heat stress (Morano et al. 2012). ROS reduces cell growth rate as well as the rate of glucose uptake, decreasing ethanol production (Woo et al. 2014). Acetic acid production is increased to stimulate NADPH production via the TCA cycle for ROS scavenging. However, acetic acid production exacerbates ROS accumulation by increasing the respiration rate and generating hydroxyl radicals via the Fenton reaction (Nicolaou et al. 2010). In addition, acetic acid may induce apoptosis (Ludovico et al. 2001). The exacerbated production of ROS increases the oxidative stress of the cell and may also lead to lipid peroxidation, protein oxidation and DNA damage (Morano et al. 2012).

High-temperature stress disrupts cell membrane integrity, increases membrane permeability and affects plasma membrane fluidity (Zhang et al. 2015). Concomitantly, membrane-associated processes, including energy generation, transport and compartmentalisation, are affected. The passive proton influx across the plasma membrane increases, resulting in dissipation of the electrochemical potential gradient. This inhibits the active uptake of essential components and affects pH and ion homeostasis and energy generation (Piper et al. 1994; Piper 1995). In addition, ROS formation is exacerbated, which alters carbon and energy metabolism to meet the energy and redox cofactor demand of the cell (Zhang et al. 2015). Furthermore, a decrease in the intracellular pH affects the activity of several cellular components (enzymes and

proteins) and could induce programmed cell death (Ludovico et al. 2001; Zhang et al. 2015). Additionally, the production of ergosterol and trehalose increase in response to heat stress (Lu et al. 2012; Zhang et al. 2015). Trehalose is essential in maintaining plasma membrane stability and ergosterol is an important cell membrane component (Zhang et al. 2015).

Increased temperatures also affect the transport activity and saturation level of soluble compounds and solvents in the cells, which might increase the accumulation of toxins, including ethanol, inside cells due to changes in the fluidity of membranes (Guan et al. 2017). Oxygen requirements increase at high temperatures, as the cells need to increase ATP generation, which is important for cell viability and resistance to ethanol (Lu et al. 2012). This increased oxygen requirement for energy generation decreases ergosterol gene expression as ergosterol production is oxygen dependent (Shobayashi et al. 2005). Ergosterol is an important component of cell membranes and a reduction in its production affects membrane stability. Membrane stability is also affected by an increase in temperature and is therefore exacerbated. Furthermore, the decreased oxygen solubility leads to high localised concentrations of oxygen within membranes, which correlate with a rapid rise in ROS due to temperature increases (Steels et al. 1994). Oxygen solubility may therefore explain the increased free-radical damage due to heat shock during aerobic conditions. In addition to generating local high concentrations of oxygen, increased temperatures lead to increased free-radical generation especially in cells grown in low oxygen concentrations.

2.14 *SACCHAROMYCES CEREVISIAE* TOLERANCES TO STRESS ASSOCIATED WITH CELLULOSIC BIOETHANOL PRODUCTION

Documentation of the essential role of *S. cerevisiae* in alcoholic fermentation goes as far back as 1858 (Liti 2015). However, this organism cannot ferment pentose sugars and is non-cellulolytic, requiring genetic engineering to produce ethanol from cellulose (Laluce et al. 2012; Della-Bianca et al. 2013; Pereira et al. 2014). Significant advances have been made towards engineering cellulolytic *S. cerevisiae* strains (Cho et al. 1999; Fujita et al. 2002; Fujita et al. 2004; Van Rooyen et al. 2005; Den Haan et al. 2007; Van Zyl et al. 2007; Jeon et al. 2009) that are also capable of fermenting pentose sugars (Hahn-Hagerdal et al. 2001; Kuyper et al. 2005; Karhumaa et al. 2006; Hahn-Hägerdal et al. 2007; Galazka et al. 2010; Demeke et al. 2013; Demeke et al. 2015). However, most of the *S. cerevisiae* strains

routinely used are laboratory strains with limited diversity and robustness. In general, industrial and natural occurring strains are more genetically complex, presenting diverse and robust characteristics (refer to sections 2.3, 2.5 and 2.6).

The current pool of industrial strains represent a small percentage of the biodiversity that occurs in nature (Steensels et al. 2014; Liti 2015). Several studies that assessed the potential of wild yeast strains for bioethanol production indicate that natural *S. cerevisiae* isolates have a higher ethanol yield in fermentation experiments simulating high gravity fermentations and artificial cellulosic hydrolysates. Indigenous strains isolated from first-generation bioethanol industrial processes often display similar performances and may even outperform the industrial strain used as inoculum during the production of these biofuels (Da Silva-Filho et al. 2005; Basso et al. 2008; Jin et al. 2013; Ramos et al. 2013; Mukherjee et al. 2014; Pereira et al. 2014; Wimalasena et al. 2014; Ruyters et al. 2015). The inherent biodiversity of natural occurring *S. cerevisiae* strains can be exploited by selecting strains with the required characteristics for specific industrial processes. The ideal *S. cerevisiae* host background to be used as CBP host for genetic improvement, therefore still requires consideration.

2.14.1 Inhibitor tolerance mechanisms

Yeast cells develop multiple inhibitor [acids, furan aldehydes phenols (AFP)] tolerance by maintaining energy production and cell homeostasis through metabolic regulation and cellular processes (Lv et al. 2014). These regulatory mechanisms are complex and require the interaction of many cellular processes. Cell division is repressed to save energy and resources for cell survival, thus lowering biomass yield (Zakrzewska et al. 2011). However, ethanol production continues, indicating that carbohydrate metabolism remains active, while the repression of glycerol and acetic acid production suggests complex metabolic flux regulation within the carbohydrate metabolism pathways.

Carbohydrate metabolism is essential for the production of energy and metabolites. During AFP exposure, the redox balance of the cells is maintained by shifting the metabolic flux into the PPP (Lv et al. 2014). AFP increase the uptake of glucose by increasing the flux through the glycolysis pathway, suggesting an increased energy demand during inhibitor stress (Santangelo 2006). Overexpression of enzymes that participate in the lower part of glycolysis increases the metabolic flux and enhances the fermentative capacity (Smits et al. 2000; Salvadó et al. 2008). The enhanced flux through

the PPP has a positive effect on redox homeostasis, improving tolerance towards furfural and acetaldehyde stress (Gorsich et al. 2006; Matsufuji et al. 2008; Cadière et al. 2011; Krüger et al. 2011).

Glycerol biosynthesis is repressed, increasing the NADH concentration required for furan aldehyde reduction (Sárvári Horváth et al. 2003; Heer et al. 2009). Alcohol dehydrogenases assist in the detoxification of inhibitory compounds and maintaining ethanol yield (Liu et al. 2004; Liu et al. 2005; Martín et al. 2007; Heer et al. 2009; Lv et al. 2014). These enzymes require the cofactors NADPH and/or NADH, therefore the metabolic flux is redistributed towards glycolysis, PPP and ethanol biosynthesis, and is diverted from glycerol and acetic acid biosynthesis (Wahlbom and Hahn-Hägerdal 2002; Liu et al. 2004; Nilsson et al. 2005; Petersson et al. 2006; Almeida et al. 2008; Liu et al. 2009). This allows cells to maintain constant levels of these key metabolites, including ATP (Lv et al. 2014).

Several resistance mechanisms are required for solvent (ethanol, inhibitors, etc.) tolerance as they disrupt the cell membrane causing cell death (Sikkema et al. 1995; Isken and De Bont 1998). Solvent-tolerant strains are able to temporarily shift the composition of membrane fatty acids or may permanently modify the ratio of saturated to unsaturated fatty acids (UFAs) to stabilise the membrane and exclude solvents (Isken and De Bont 1998; Dyer et al. 2002; Dunlop 2011). Shifting the UFAs from *cis* to *trans* decreases membrane fluidity, thus increasing solvent tolerance (Holtwick et al. 1997; Junker and Ramos 1999; Kiran et al. 2004). However, a decrease in membrane permeability traps the molecules within the cell, therefore a combination of tolerance mechanisms is needed.

Regulation of protein synthesis increases tolerance against AFP stress and helps with the redistribution of energy to processes essential for the survival and reproduction of cells in the presence of these compounds (Lv et al. 2014). Protein synthesis is downregulated, followed by the downregulation of the expression of protein folding chaperones, thus increasing tolerance by minimising energy expenditure and alleviating protein-folding stress.

Saccharomyces cerevisiae contains multidrug-resistant (MDR) efflux pumps that belong to the ATP-binding cassette (ABC) transporter family and the major facilitator superfamily (MFS). These efflux pumps confer resistance to a wide range of compounds

and metal ions (Mahé et al. 1996; Zgurskaya 2002; Ambudkar et al. 2003; Pumbwe et al. 2006; Panwar et al. 2008) by recognising and exporting these compounds from the cell using the proton motive force (Dunlop 2011). The ability of MDR pumps to mediate the efflux of toxic compounds depend on the permeability of the membrane to the compound (Eytan et al. 1996). Furthermore, drug-efflux pumps are located on the plasma membrane (Panwar et al. 2008) and changes in the membrane lipid composition have adverse effects on these proteins (Kaur and Bachhawat 1999).

2.14.2 Temperature tolerance mechanisms

Heat shock has adverse effects on the post-translational modification of proteins, causing the accumulation of unfolded proteins (Morano et al. 2012; Wohlbach et al. 2014). The heat shock response (HSR) is activated to protect proteins against degradation by activating the synthesis of molecular chaperones. During stress conditions, heat shock proteins (HSPs) prevent protein aggregation and assist with the refolding and stabilisation of denatured proteins to maintain functional conformation. HSPs also protect cells against apoptosis induced by oxidative stress, heat shock, toxins and cellular damage (Parsell and Lindquist 1993; Piper 1995; Balakumar and Arasaratnam 2012). Gene expression of HSPs is therefore upregulated when cells are exposed to various stresses, including heat shock, glucose starvation, oxidative stress, toxins, cellular damage and solvents (Parsell and Lindquist 1993; Piper 1995; Ding et al. 2009; Dunlop 2011; Balakumar and Arasaratnam 2012). In addition, HSPs downregulate the stress-activation of plasma membrane H^+-ATPase to maintain energy reserves (Piper et al. 1994; Piper et al. 1997).

Yeast cells adjust their membrane lipid compositions in response to various stresses, including heat and oxidative stress. This prevents and/or minimise the ability of harmful components to enter the cell, thus shielding the cellular components against damage and maintaining cellular function and viability (Chatterjee et al. 2000; You et al. 2003). Trehalose has a protective effect on membranes (Peres and Laluce 1998) and is used as a protection mechanism against several environmental and industrial stressors, including thermo-, osmo- and ethanol tolerance (Neves and Francois 1992; Sharma 1997; Hounsa et al. 1998; Singer and Lindquist 1998). Trehalose acts as a membrane protectant by stabilising membranes and therefore prevents membrane fluidisation during increased temperature (Mansure et al. 1997). Trehalose cooperatively promotes

misfolded-protein refolding processes, thereby maintaining structure and cellular function and preventing protein aggregation (Singer and Lindquist 1998; Swan and Watson 1998; Gasch et al. 2000; Ding et al. 2009; Delic et al. 2012).

L-proline also acts as an osmoprotectant by protecting cells from damage by desiccation, extreme temperatures, or oxidative stress. L-proline enhances protein and membrane stability during low water activity and high temperatures, and inhibits aggregation during protein refolding, thereby improving thermo- and ethanol tolerance (Morita et al. 2003; Terao et al. 2003; Takagi et al. 2005).

2.14.3 Environmental stress response (ESR)

Saccharomyces cerevisiae encounters many environmental stresses during industrial alcoholic fermentations, including osmotic stress, oxidation, low pH and high ethanol concentrations, which restrict yeast growth and viability (Blieck et al. 2007; Laopaiboon et al. 2009; Zhao and Bai 2009). Environmental stress affects microbial cell viability by changing the intracellular environment in terms of pH, water activity and redox potential and has a denaturing effect on proteins and other cellular components. *Saccharomyces cerevisiae* has developed tolerance mechanisms to multiple simultaneous stresses by regulating the expression of genes involved in different cellular metabolic processes, thus adjusting these processes to overcome stress conditions (Gasch et al. 2000; Tirosh et al. 2011).

2.14.3.1 General stress response

The general stress response (GSR) protects the cell against a number of environmental stresses, including oxidative, pH, heat and osmotic stresses (Martinez-Pastor et al. 1996; Schmitt and McEntee 1996; Hohmann 2002) and involves the induction of more than 200 genes in response to these stresses (Gasch et al. 2000; Causton et al. 2001). These genes contain a general stress-response element (STRE) that binds two TFs, MSN2 and MSN4 (Marchler et al. 1993; Martinez-Pastor et al. 1996; Schmitt and McEntee 1996; Wohlbach et al. 2014). TFs play an essential role in the regulation of gene expression and several have been identified as key regulators of environmental and chemical stress tolerance (Natarajan et al. 2001; Yale and Bohnert 2001; Hahn et al. 2006; Ma and Liu 2010b).

2.14.3.2 Transcription factors

Transcription factors (TFs) have multiple binding sites and regulate each other and themselves to form regulatory networks that allow an integrated response to environmental stress. Binding of TFs allows for the differential expression of genes that are important in generating phenotypic diversity as discussed in section 2.6. Several TFs are essential in regulating the various stress responses required for environmental adaptation, including the general stress response (TF MSN2/4), oxidative stress response (TF YAP), nutrient stress response (TF GCN), heat shock response (TF HSF), proteasome degradation pathway (TF RPN4) and drug resistance (TF PDR3).

Multicopy suppressor of SNF1 mutation (MSN2/4)

The STREs contained in general stress response genes are regulated by the TFs MSN2 and MSN4. During stress conditions, MSN2 migrates to the nucleus to activate transcription of several genes (Boy-Marcotte et al. 1998; Görner et al. 1998; Garreau et al. 2000; Gorner et al. 2002). The stress response is rapid, but quickly attenuated due to MSN2 degradation. MSN2/4 induction of TFs occurs in response to various stress conditions, including heat shock, osmotic stress and carbon-source starvation, and is also implicated in diauxic growth (Boy-Marcotte et al. 1998; Görner et al. 1998).

Ethanol tolerance genes (*HSP31, HSP32*, *HSP150* and *GND2*) have protein-binding motifs for TFs MSN2/4, YAP1 and HSF1, with several sharing the general binding sequence for all three TFs, indicating a key regulatory role of MSN2/4 in ethanol tolerance (Ma and Liu 2010a). Additionally, all three TFs show distinct expression patterns upon exposure to ethanol. *HSP32* is co-regulated by HSF1; *HSP150* is co-regulated by YAP1; and *GND2* and *HSP31* are co-regulated by both HSF1 and YAP1 (Ma and Liu 2010a).

The general MSN2/MSN4-mediated stress response (Rep et al. 2000) also induces genes involved in the protection from oxidative damage (*CTT1*, *DAK1, GLO1, GRE2, GRX1, TTR1, YBL064C, YBR014C, YDR453C* and *YGL157W*) and/or to altered redox metabolism (*ALD2*, *ALD3*, *ALD4*, *ALD6*, *ARA1, CYB2, CYC7, GRE2, GRE3, GND2, MCR1, YAL061W*, *YGL157W*, *YML131W, YMR090W* and *ZTA1*) (Rep et al. 2000). The production of many of these proteins is stimulated by oxidative (Godon et al. 1998) and osmotic stress (Blomberg 1995; Norbeck and Blomberg 1997). In contrast, salt tolerance is due to an increase in the biosynthesis of glycerol mediated by MSN2 in a HOG1-dependent manner (Nevitt et al. 2004).

Yeast activator protein (YAP)

The YAP family of TFs co-regulates the transcription of several genes required in a wide range of processes including amino acid metabolism, DNA repair, chaperones, GSR, HSR, and pleiotrophic drug resistance (PDR). YAP1 is the main oxidative stress regulator, performing as a sensor for oxidative molecules, inducing genes required for antioxidant defence and thiol redox control (Delaunay et al. 2002; Rodrigues-Pousada et al. 2004). This TF recognises the YAP1 response elements (YREs) in the promoter region of several genes (Harbison et al. 2004; Fernandes et al. 1997; Lee et al. 2002; Haugen et al. 2004; Dubacq et al. 2006). These include oxidoreductase enzymes with aldehyde reductase activity, including the alcohol dehydrogenases (ADH1, ADH6 and ADH7), aldehyde dehydrogenase (ALD4), aldehyde reductase (ARI1), methylglyoxal reductase (GRE2 and GRE3) and mitochondrial transaldolase (TAL1) (Liu 2011). Exposure to furan aldehydes upregulates the transcription of *YAP1* (Ma and Liu 2010b), which leads to an upregulation of the genes regulated by this TF (Heer et al. 2009; Ma and Liu 2010b) to convert the furan aldehydes to their less toxic counterparts, thus enabling the organism to survive inhibitor exposure. YAP1 therefore plays an important role in inhibitor toxicity, as oxidoreductase is required for furan aldehyde detoxification. Furthermore, *YAP1* transcription has also been implicated in short-term adaptation to acidic pH (Petersson et al. 2006).

YAP2 induces transcription of *FRM2*, encoding a protein homologous to nitroreductase (Rodrigues-Pousada et al. 2004). In addition, YAP2 confers resistance to various chemical compounds including phenanthroline, cadmium, cerulenin and cycloheximide (Bossier et al. 1993; Wu et al. 1993), with YAP1 also mediating cadmium resistance. YAP8 (ACR1) confers resistance to arsenic compounds by positively regulating *ACR2* (encoding an arsenate-reductase) and *ACR3* (encoding a plasmamembrane arsenite efflux protein) (Wysocki et al. 1997; Mukhopadhyay and Rosen 1998; Mukhopadhyay et al. 2000).

YAP4 (HAL6) and YAP6 (HAL7) have been implicated in oxidative, heat and osmotic tolerance (Gasch et al. 2000; Posas et al. 2000; Rep et al. 2000). Two genes involved in glycerol biosynthesis, *GCY1*, encoding a putative glycerol dehydrogenase, and *GPP2*, encoding a NAD-dependent glycerol-3-phosphate phosphatase, are induced by YAP4 during osmotic stress (Rodrigues-Pousada et al. 2004). Furthermore, *DCS2* - a homolog to the *DCS1* encoded decapping enzyme - is also regulated by YAP4. DCS1 is an inhibitor of trehalase activity (De Mesquita et al. 2003), indicating a role in osmotolerance as

trehalose provides protection against dehydration and desiccation (Singer and Lindquist 1998; Sun and Davidson 1998).

General control nonderepressible (GCN)

GCN4 is a major regulator of gene expression, allowing for a wide range of transcriptional and signalling responses during nutrient starvation and cell stress, with 94 of the genes induced during the ESR being GCN4 targets (Hinnebusch 1984; Rolfes and Hinnebusch 1993; Yang et al. 2000; Natarajan et al. 2001). Genes in all amino acid biosynthetic pathways (except cysteine) and purine biosynthesis (78 genes), genes encoding amino acid precursors (*ACO1, ACO2, IDH1, IDH2* and *IDP1*), genes encoding amino acid transporters (*AGP1, BAP2, CAN1, GAP1, LYP1* and *MUP3*) and protein phosphatase regulatory subunits, and genes encoding precursors for glutamate, glutamine or cysteine biosynthesis (*GIP1, GLC7, PTP1* and *SAP4*) have been identified as targets for GCN4 (Natarajan et al. 2001).

Several additional genes contain the GCN4 binding site (UAS_{GCRE}) in their promoters, coding region and untranslated regions, or are indirectly regulated by GCN4 through TFs that are induced by GCN4 (Natarajan et al. 2001). These include genes involved in vitamin and cofactor biosynthesis (*ADE3, BIO3, BIO4, BIO5, BNA1, FOL2, PDX3, RIB1, RIB3, RIB5, SNO1, SNZ1, YBL098W, YDR531W, YEL029C* and *YFR047C*), peroxisomal biogenesis (*PEX1, PEX5, PEX11, PEX14, PEX21* and *PXA2*), mitochondrial transport (*ARG11, CRC1, OAC1, YER053C, YHM1, YMC1, YMC2, YOR222W, YPR021C* and *YPR128C*), autophagy (*APG1, APG13* and *APG14*), glycogen homeostasis (*GDB1, GLC3, GLG1, GSY1, GSY2* and *GPH1*), and genes encoding vacuolar proteases (*AAP1* and *LAP4*). GCN4 also induces the transcription of regulatory genes, including genes encoding protein kinases (APG1, DBF20, NPR1, STE11, TPK1 and TPK2) and TFs (26 genes).

Heat shock transcription factor (HSF)

Heat shock factor (HSF), the principal regulator of the HSR, monitors the translational status of cells through a ribosomal quality control-mediated translation-stress signal. HSF activates the expression of multiple genes in response to a variety of diverse stresses, thereby playing a crucial role in regulating cellular homeostasis (Morimoto 1998; Pirkkala et al. 2001; Hahn et al. 2006; Morano et al. 2012). The principal role of HSF is the activation of HSP transcription that function as molecular chaperones in protein folding,

stabilisation, activation, trafficking and degradation (Parsell and Lindquist 1993; Morimoto 1998; Pirkkala et al. 2001).

In addition, HSF also binds to genes encoding TFs, including the RPN4, PDR3 and YAP1 TFs (Lee et al. 2002; Harbison et al. 2004; Hahn et al. 2006; Workman et al. 2006). This enables the interaction of several stress-responsive regulatory pathways, allowing the formation of networks that play an important role in protection, adaptation and metabolic remodelling in cells exposed to stress. HSF regulates *RPN4* expression through a feed-forward loop by direct (*RPN4* promoter) and indirect (regulating expression of TF PDR3) binding (Lee et al. 2002; Shen-Orr et al. 2002). The expression of *HSF1* is enhanced by HMF, which regulates the expression of *HSP26* and *SSA4* (Harbison et al. 2004; Ferguson et al. 2005; Ma and Liu 2010b). Furan aldehyde exposure induces the transcription of these proteins to counteract the protein damage caused by the inhibitory compounds (Liu 2011).

Regulatory particle non-ATPase (RPN4)

RPN4 is a TF that stimulates the expression of proteasome genes. During non-stressed conditions, RPN4 is required for the expression of proteasome subunits genes (Xie and Varshavsky 2001) and several genes involved in DNA repair, including *MAG1*, *DDI1*, and *RAD23* (Jelinsky et al. 2000; Harbison et al. 2004; Zhu and Xiao 2004). RPN4 has a short lifespan and is also degraded by the 26S proteasome, creating a negative feedback control mechanism (Xie and Varshavsky 2001). This regulation mechanism of *RPN4* expression is essential in modulating cellular RPN4 function. Furthermore, the *RPN4* promoter contains regulatory elements for the binding and action of three TFs (HSF, PDR1/3 and YAP1), and functions as a feedback regulator of YAP1 and PDR1 (Devaux et al. 2002; Hahn et al. 2004; Hahn et al. 2006; Salin et al. 2008). This co-ordinated regulation of *RPN4* expression by multiple regulators allows for flexible expression of *RPN4* and its targets in response to environmental and physiological stresses including heat, chemical and oxidative stress (Hahn et al. 2006). In addition, overlap in transcriptional regulatory networks connects various stress responses. Furthermore, heat-induced expression of RPN4 leads to a time delay in the expression of RPN4 targets, allowing a temporal control mechanism for proteasome synthesis during stress conditions that could result in irreversible protein damage (Hahn et al. 2006). The heat shock element (HSE) is the main regulatory element for heat- and methyl methanesulphonate (MMS) induced expression

of *RPN4* and is mediated by HSF through binding to the HSE within the *RPN4* promoter (Hahn et al. 2006). MMS damages DNA and proteins and leads to the activation of the TFs MEC1 and YAP1 (Gasch et al. 2000).

Pleiotrophic drug resistance (PDR3)

PDR3 is a transcriptional activator of the PDR network and has a role in response to drugs and organic solvents. It regulates the expression of ABC transporters through binding to the DNA binding site of PDRs, the PDR responsive element (PDRE). PDREs contribute to heat shock and MMS induction of *RPN4,* reflecting the HSF-mediated regulation of *PDR3* expression. PDR1/3 may play a role in activation of *RPN4* during specific physiological or environmental stress conditions, such as mitochondrial dysfunction that regulates PDR3-mediated responses. PDR3 also directly activates *RPN4* gene expression (Devaux et al. 2002)

PDR1/3 binds to the promoters of *MAG1* and *DDI1* (Zhu and Mivechi 1999; Jelinsky et al. 2000) and regulate *MAG1* directly or indirectly through *RPN4*, forming a feed-forward regulatory loop. These proteins are involved in DNA damage; *MAG1* encodes a DNA glycosylase (Chen et al. 1990) that catalyses the first step in the base excision repair pathway and protects cells from DNA-alkylating agents (Zhu and Xiao 2004; Fu et al. 2008). *DDI1* encodes a ubiquitin-related protein and is involved in DNA-damage cell-cycle checkpoint (Clarke et al. 2001; Zhu and Xiao 2004). Concomitantly, ethanol tolerant strains showed enhanced *DDI1* expression (Ma and Liu 2010a).

Several HSF targets (*AHP1*, *HSP12*, *HSP26, NCE102, SNG2*, *SNQ2* and *SPI1*) are also targets of PDR3 (Hahn et al. 2004; Onda et al. 2004), suggesting a role for HSF in MDR. *SNQ2*, an ABC transporter, and *RPN4* are regulated by three TFs, HSF, YAP1 and PDR3 (Devaux et al. 2002; Hahn et al. 2004). During stress conditions, HSF and YAP1 activate *RPN4* and *SNQ2* through direct binding, while also allowing indirect regulation through PDR3. In addition, PDR3 positively autoregulates its own expression. This complex regulatory network guarantees the expression of *RPN4* and *SNQ2* during specific stress conditions, including DNA damage and oxidative stress. This regulatory overlap among HSF, YAP1 and PDR3 indicates a tightly co-ordinated regulatory and functional interdependent organisation between the stress responses and MDR (Hahn et al. 2006).

2.14.3.3 Heat shock response

HSP genes that are significantly upregulated in response to environmental stress, include those involved in protein catabolic processes, ubiquitin-dependent protein catabolic processes, proteolysis, protein folding and stress-related genes. Upregulated genes involved in protein folding and refolding (*HSP10, HSP26, HSP60, HSP78, HSP82, HSP104, SSE1, SSE2* and *SSA1-4*) allow for the correct folding of denatured proteins (Boy-Marcotte et al. 1999; Yamamoto et al. 2008; Ismail et al. 2013).

During ethanol exposure, HSP12, HSP26, HSP42, HSP78 and HSP82 prevent proteins from aggregating, HSP104 disassembles protein aggregates, and HSP150 is involved in cell wall and structural molecule activity. HSP31 and HSP32 function as a chaperone and cysteine protease, respectively, and are involved in protein binding, peptidase and hydrolase activities with additional functions in cell component and biological processes (Ma and Liu 2010a), whereas HSP82 activates cellular regulatory and signalling proteins, such as TFs and regulatory kinases (McClellan et al. 2007). Significantly enhanced expression of *HSP30*, *HSP31* and *HSP150* was observed in an ethanol tolerant strain (Ma and Liu 2010a). TFs MSN2/4, HSF1 and YAP1 also regulate these HSP genes (see MSN2/4 section). HSP genes upregulated in response to osmotic stress (*HSP12*, *HSP26*, *HSP42*, *HSP104*, *DDR2*, *DDR48*, *CTT1, SSA3*, *SSA4* and *SSE2*) are involved in chaperone and protective functions (Rep et al. 2000), whereas increased temperature induces the expression of *HSP82*, *HSP104* and *SSA4*. Transcription of most of these genes is dependent on the general MSN2/4-mediated stress response (Rep et al. 2000).

2.14.3.4 Oxidative stress response

The oxidative stress response protects the cell against the oxidative effects of free radicals, including ROS. This may be through enzymatic (antioxidant systems) and non-enzymatic mechanisms (cofactor recycling and generation through oxidoreductases mechanisms) (Herrero et al. 2008; Ramos et al. 2013). Oxidoreductases are induced by various environmental factors and provide protection against osmotic, ionic, oxidative and heat shock stresses (Grant 2001; Garrido and Grant 2002; Vogel et al. 2011). The interconversion of pyridine-nucleotide cofactors ($NADH/NAD^+$ and $NADPH/NADP^+$) is essential to maintain redox metabolism and plays a key role in the oxidative stress response. NADH is required to convert pyruvate into CO_2 and ethanol during respiration

and fermentation, whereas NADPH is required for the synthesis for amino acids and nucleotides.

The oxidative phase of the PPP is the main source of NADPH production in yeast (Liu 2011). During oxidative stress, the cellular enzymes involved in the PPP are induced while glycolysis enzymes are repressed, indicating an increased demand for NAD(P)H (Godon et al. 1998). NAD(P)H enhances yeast tolerance toward oxidative, acidic and chemical stresses (Grant 2008; Krüger et al. 2011). The PPP generates reducing equivalents and various sugar molecules required in the biosynthesis of nucleic and amino acids. Redistribution of the carbohydrate metabolic flux into the PPP therefore increases tolerance towards oxidative stress (Ralser et al. 2007). In addition, the resistance mechanisms against furfural and HMF include the ability of organisms to grow and metabolise these compounds by overexpressing aldehyde reductases that are NADH- or NADPH-dependent (Petrovič 2015).

The specific fermentation conditions also play a role in the oxidative response. Aerobically grown cells are more stress tolerant than anaerobically grown cells because of the induction of antioxidant enzyme systems. Anaerobically grown cells obtain energy via glycolysis, whereas the aerobically grown cells, although subject to catabolite repression, obtain energy via glycolysis and respiration (Steels et al. 1994). In the presence of oxygen and low sugar availability, ethanol is utilised as a carbon source, but this cannot occur during anaerobic conditions (Lin et al. 2012). When sugar is limited, yeasts switch from a fermentative metabolism, depending mainly on glycolysis and producing ethanol, to a respiratory metabolism where the ethanol is consumed via the TCA and glyoxylate cycles and the mitochondrial electron transport chain (Rolland et al. 2002). The specific molecular mechanisms involved in the oxidative stress response are discussed in the metabolism section (2.14.3.8).

2.14.3.5 Role of intracellular pH

Intracellular pH is important in determining the fermentation pathway used during anaerobic ethanol production and changes in the operational pH may induce a change in the main fermentation pathway leading to by-product formation such as acetic and butyric acid, thereby reducing the efficiency of ethanol fermentation (Lin et al. 2012). In the presence of stress, the activity and amount of H^+-ATPases increase. The H^+-ATPases are responsible for maintaining ion homeostasis in the cytoplasm, subsequently affecting

the permeability of the yeast membrane (Furukawa et al. 2004). The increased ATPase activity counteracts the proton influx induced by ethanol and activated by a change in lipid composition, thus increasing ethanol tolerance (Cartwright et al. 1987). This counteracts the disturbed membrane permeability and electron chemical proton gradient resulting from ethanol exposure (Fernandes and Sá-Correia 2003).

Low pH conditions cause acidification, decreasing the intracellular pH, which activates the plasma membrane ATPases and MDR transporters to eliminate intracellular H^+ (Calahorra et al. 1987). ATPases are responsible for the efflux of protons, whereas the MDR transporters pump anions out of the cell. These membrane transport mechanisms require ATP, thus increasing the energy demand (Piotrowski et al. 2014; Caspeta et al. 2015). As discussed in section 2.12.1, weak acids are predominantly in their undissociated form at low external pH and can diffuse across the plasma membrane. Inside the cytosol, which is pH neutral, these acids dissociate and the subsequent energy-dependent export of protons and anions leads to a futile ATP-spending cycle (Pampulha and Loureiro-Dias 2000). Low pH affects cell growth and induces the general stress response, including the downregulation of transcription and protein synthesis due to protein kinase A (PKA) based glucose signalling (De Melo et al. 2010). A decrease in pH also induces adaptations in cell wall composition and structure by increasing chitinase levels, thereby decreasing the cell wall chitin level (Cabib et al. 1989). The specific molecular mechanisms involved in maintaining intracellular pH are addressed in the metabolism section (2.14.3.8).

2.14.3.6 Osmotic stress response

Two signal transduction pathways are implicated in regulating processes required for osmotic adjustment and ion homeostasis. The high osmolarity glycerol (HOG) pathway is responsible for the control of osmotic adaptation, whereas the calcineurin pathway regulates ion homeostasis. The key regulatory protein of the HOG pathway is a mitogen-activated protein kinase (MAPK). This pathway is activated in response to hypertonic stress detected by either of two osmosensing proteins, SLN1 or SHO1 (Varela and Mager 1996; Gustin et al. 1998). Signalling transduced through the HOG cascade activates transcription of several stress-responsive genes necessary for osmotic adaptation, including glycerol-3-phosphate dehydrogenase (*GPD1*) (Gustin et al. 1998; Posas et al.

2000; Rep et al. 2000; Yale and Bohnert 2001). GPD1 catalyses a crucial step in the biosynthesis of glycerol, which is the main osmolyte in yeast cells.

The calcineurin signal pathway mediates cellular sodium, potassium and calcium ion homeostasis using calcineurin, a Ca^{2+}/calmodulin-dependent type 2B phosphatase, as intermediate (Nakamura et al. 1993; Mendoza et al. 1994). Hypersaline stress activates calmodulin via Ca^{2+}, which subsequently activates calcineurin (Nakamura et al. 1993; Mendoza et al. 1994). Signalling through calcineurin regulates the P-type ATPase that is responsible for Na^{+} efflux across the plasma membrane, and endomembrane-localised Ca^{2+}-ATPase pumps that mediate Ca^{2+} homeostasis.

Modelling of the cell surface is controlled by the ability of the cytoplasm to expand (Heinisch et al. 1999). Osmotic shock causes cells to shrink, allowing growth at a smaller size in high osmolarity medium, thereby affecting processes related to cell surface assembly. Genes encoding proteins with functions related to the cell surface and cell wall formation (*CWP1*, *ECM37*, *SPI1*, *SPS100*, *YBR056W, YCP1* and *YLR042C*) are therefore induced by osmotic shock (Rep et al. 2000). Ergosterol is an important cell membrane component and ergosterol metabolism therefore plays an important role in osmotic stress and ethanol exposure. Ethanol exposure upregulates the expression of genes involved in ergosterol metabolism (*ERG20, ERG24* and *ERG26*) (Ma and Liu 2010a), whereas osmotic stress represses sterol production (repression of *ERG3*, *ERG6*, *ERG11*, *ERG25* and *OYE2*) (Rep et al. 2000). In addition, cell surface modelling is controlled by the MAPK pathway, specifically protein kinase C (PKC) (Gustin et al. 1998; Heinisch et al. 1999), which is stimulated by hypo-osmotic shock (Davenport et al. 1995). The HOG and MAPK pathways appear to control each other (Davenport et al. 1995), thus stimulation of the HOG pathway may affect cell surface modelling indirectly via its effect on this pathway. Osmotic shock also affects the expression of genes involved in vacuolar biogenesis (*PRB1*, *VPS36*, *YGR066C* and *YHR138C*) because of the involvement of this organelle in cellular water and ion homeostasis.

2.14.3.7 Transport

The main mechanism mediating MDR in yeast involves transporters that export or compartmentalise structurally and functionally unrelated compounds by activating the transcription of genes encoding ABC and MFS membrane transporters (Devaux et al. 2002; Kolaczkowska et al. 2002). The mitochondrial ABC transporter (MDL1) is involved

in oxidative stress (Chloupková et al. 2003), whereas the vacuolar ABC transporters (ScYcf1p and ScBpt1p) help in cellular detoxification by sequestering toxic compounds (Szczypka et al. 1994; Petrovic et al. 2000; Klein et al. 2002; Sharma et al. 2002).

In *S. cerevisiae*, MDR is regulated by six TFs (PDR1, PDR3, PDR8, YKL222C, YRM1 and YRR1) (Devaux et al. 2002; Kolaczkowska et al. 2002; Le Crom et al. 2002; Onda et al. 2004). These TFs share overlapping target genes, including *SNQ2* and *YOR1*, in addition to regulating specific individual gene sets. YAP1 is also involved in MDR by activating the expression of ABC transporter genes (*SNQ2* and *YCF1*) and MFS transporter genes (*ATR1* and *FLR1*) (Alarco et al. 1997; Nguyên et al. 2001; Tenreiro et al. 2001). Furthermore, YAP1-mediated diazaborine resistance is dependent on PDR1/3, indicating a functional interaction among these TFs (Wendler et al. 1997).

As discussed in the PDR section (2.14.3.2), *PDR* genes encode plasma membrane proteins that operate as ABC transporters. These transporters mediate membrane translocation of ions, metabolites and other substrates from the intracellular environment (Liu 2011). The functions of the PDR gene family is regulated by PDR1/3 and other co-regulatory genes including *YAP1* and *HSF1*. Binding of PDR1/3 to these PDREs regulate the expression of several genes (Mamnun et al. 2002; Jungwirth and Kuchler 2006), including genes involved in viability, adaptation to chemical stress, transport, lipid composition and DNA repair (Tomitori et al. 2001; Teixeira and Sá-Correia 2002).

Genes of the PDR family are also induced by various chemical stress conditions, including furan aldehydes and ethanol exposure (Mamnun et al. 2002; Jungwirth and Kuchler 2006; Alriksson et al. 2010; Ma and Liu 2010a). PDR5, PDR15, SNQ2 and YOR1 are involved in the export of xenobiotic compounds and endogenous toxic metabolites, whereas TPO1 and TPO4 are involved in the export of polyamines. Induction of these PDR genes could prevent the influx of HMF into the cytoplasm and other important organelles by remodelling the membranes, thus increasing tolerance to HMF (Liu 2011). PDR1/3 are also directly implicated in membrane adaptability by activating the transcription of genes for lipid metabolism (Devaux et al. 2002; Kolaczkowska et al. 2002). For instance, PDR genes *RSB1* and *ICT1* are involved in phospholipid synthesis, transportation of membrane structures and tolerance to organic solvents (Miura et al. 2000; Ghosh et al. 2008). Genes significantly up-regulated in response to ethanol exposure (*GRE2*, *PDR15, TPO1* and *YMR102C)* have more than one PDRE (Ma and Liu 2010a).

2.14.3.8 Metabolism

Environmental stress resistance mechanisms include metabolic rearrangements such as carbohydrate metabolism, protein synthesis, ergosterol biosynthesis, glutathione transport and trehalose catabolism (Swan and Watson 1998; Liu 2011; Delic et al. 2012; Petrovič 2015). Regulation of cellular metabolism facilitates adaptation to the specific environment and maintains cell survival (Allen et al. 2010).

Carbohydrate metabolism is required for energy production as well as various metabolites, and therefore has a critical effect on cellular processes (Gasch 2002). Cells exposed to osmotic, acidic and thermal stress display an increase in glycolytic flux (Salvadó et al. 2008). Carbohydrate metabolism is shifted to combat oxidative stress or to assist in energy generation to maintain cell viability. Furthermore, *S. cerevisiae* has isogenes for many enzymes involved in sugar metabolism that are preferentially expressed (Rep et al. 2000). Poorly expressed isogenes are induced after osmotic shock, indicating a switch of the isoform expression pattern (Akhtar et al. 1997; Blomberg 1997). These include genes for sugar transporters (*HXT1*, *HXT5*, *STL1*, *YBR241C* and *YGL104C*), glucokinases (*GLK1* and *YDR516C*), enzymes in the PPP (*GND1* and *GND2*), transaldolases (*TAL1*, *TKL2*, *YGR043C* and *XKS1*) and genes for enzymes involved in glycolysis (enolase and glyceraldehyde-3-phosphate dehydrogenase) (Rep et al. 2000). The isogenic expression pattern of *S. cerevisiae* reflects an adaptation to a variable environment where certain isoforms are more stable during stress conditions (Rep et al. 2000).

Exposure to furan aldehydes inhibits yeast growth and reduces transcription of genes involved in glycolysis and the PPP (Liu 2011), resulting in a decrease of ATP, NAD(P)H and intermediate metabolites required for cell growth and reproduction (Wahlbom and Hahn-Hägerdal 2002). Inhibitor tolerant strains have an induced expression of glycolysis (*HXK1*, *HXK2*, *GLK1* and *TDH1*), and PPP (*GND1*, *GND2*, *SOL3* and *ZWF1*) genes and a reduced expression of the glycolytic enzyme phosphoglucose isomerase *PGI* (Liu 2011), thus driving glucose metabolism toward the PPP with a concomitant regeneration of NAD(P)H cofactors required for the detoxification of the inhibitors.

Genes involved in glycolysis and the PPP are upregulated during ethanol exposure, including (*ADH1, ADH2, ADH3, ADH7, ALD4, GPM2, IRC15, NQM1, PGM2, SFA1, SOL4* and *YDR248C*) (Ma and Liu 2010a). Increased temperature affects the expression of genes in

the glycolytic pathway. For example, expression of *ADH1* and *CDC19* is significantly induced, whereas the expression of *ADH2* is repressed. Acetic acid stress also increases the expression of various genes involved in glycolysis, the Krebs cycle and ATP synthesis (Mira et al. 2010a; Mira et al. 2010b). Some enhanced carbohydrate metabolism genes (*ADH1*, *ALD4, GLK1, GND2, GPM2, HXK1, SOL4* and *TDH1*) implicated in inhibitor and ethanol tolerance are also involved in mitochondria functions (Ma and Liu 2010a).

Trehalose metabolism is affected in response to environmental stress. Ethanol exposure increases transcription of trehalose metabolism genes (*ATH1*, *GSY2, NTH1*, *NTH2*, *TPS1* and *TSL1*) (Ma and Liu 2010a). However, increased temperature downregulates the expression of genes involved in trehalose metabolism (*NTH1, TPS1* and *TPS2*). Osmotic stress induces the production and degradation of genes encoding enzymes in trehalose (*NTH1, TPS1*, *TPS2*, *TSL1* and *UGP1*) and glycogen (*GLC3, GSY1*, *GSY2* and *PGM2*) metabolism (Nwaka et al. 1995; Parrou et al. 1997; Zähringer et al. 1997; Parrou et al. 1999). This leads to a futile cycle of trehalose and glycogen production and degradation.

Glycerol metabolism plays a role in the ESR. During osmotic stress, the expression of glycerol metabolism genes is induced, including *GLO1* and *DAK1* (Ma and Liu 2010a; Rep et al. 2000). Different isoforms of glycerol-3-phosphate dehydrogenase (*GPD1* and *GPD2*) and glycerol-3-phosphatase (*GPP1* and *GPP2*) are also induced by osmotic shock (Albertyn et al. 1994; Norbeck et al. 1996; Rep et al. 1999b; Rep et al. 1999a). *GPD2* is induced during anaerobic conditions when glycerol production is required for cellular redox regulation (Ansell et al. 1997). *GPH1*, a gene involved in glycogen catabolism is also regulated by stress response elements and the HOG-MAPK pathway (Ma and Liu 2010a). In addition, ethanol exposure increases the expression of genes involved in glycerol metabolism (*DAK1*, *GCY1*, *GPD1, GUP1* and *GUP2*). GLO1 and DAK1 are involved in the detoxification of by-products of glycerol production (Norbeck and Blomberg 1997; Inoue et al. 1998). In addition, DAK1 (dihydroxyacetone kinase) forms part of the glycerol degradation pathway to regulate the cellular glycerol content or to prepare the cell for glycerol degradation (Norbeck and Blomberg 1997).

RNA metabolism is implicated in environmental stress tolerance by allowing the redirection of the translational machinery toward the preferential production of stress-related proteins, specifically HSPs (Bond 2006). The spliceosome consists of several small nuclear ribonucleoproteins (snRNPs) that are required for mRNA splicing, and is

extremely sensitive to thermal stress (Bracken and Bond 1999). Yang et al. (2013) identified two causative genes (*PRP42* and *SMD2*) involved in thermotolerance that are involved in pre-mRNA splicing, suggesting an essential role for RNA processing in conferring thermotolerance. PRP42 is an essential protein for U1 snRNP biogenesis, whereas SMD2 is part of the spliceosomal U1, U2, U4 and U5 snRNPs that function in pre-mRNA splicing.

Nucleotide and amino acid metabolism are affected by chemical and environmental stress. Genes involved in the catabolism of certain amino acids (*ARO9*, *ARO10*, *PUT4* and *YMR250W*) are induced, while genes in amino acid and nucleotide biosynthesis (*CYS4*, *DPH5, FUR1, HOM3, HPT1, MET6, MET25, PRO2* and *SAM1*) are repressed (Rep et al. 2000; Ma and Liu 2010a). The regulatory genes (*ARG80*, *ARG81* and *GCN4*) involved in arginine biosynthesis are downregulated during inhibitor exposure, including transcription of the genes regulated by these TFs (*ARG1*, *ARG3*, *ARG4*, *ARG5*, *ARG6*, *ARG7* and *ARG8*). Concomitantly, the transcription of an enzyme involved in arginine catabolism (*CAR1*), is upregulated (De Rijcke et al. 1992; Natarajan et al. 2001; Ma and Liu 2010b). Genes required in lysine biosynthesis (*LYS4*, *LYS14* and *LYS20*) are also repressed, whereas genes involved in proline (*PUT1* and *PUT2*), serine (*CHA1*) and alanine (*ALT1*) catabolism are induced (Ma and Liu 2010b). Genes encoding enzymes in methionine biosynthesis are strongly repressed as sulfhydryl groups are required for the production of glutaredoxin and thioredoxin (Norbeck and Blomberg 1997). Ethanol exposure represses all genes involved in tryptophan biosynthesis, except *TRP5*, while inducing expression of *PUT1* involved in proline biosynthesis (Ma and Liu 2010a). This altered gene expression leads to an increase in amino acid catabolism and a reduction in amino acid biosynthesis. Amino acids metabolism pathways are integrated with the TCA cycle, thus providing a mechanism for ATP regeneration. These changes are also responsible for the temporary growth arrest observed in cells during stress conditions.

Protein synthesis is also affected by environmental stress. Genes encoding ribosomal proteins and proteins involved in translation (*ASC1, EFB1, EFT1/EFT2, EGD2, GAR1, ILS1, NOP1, PAB1*, *SIK1, SNU13, SSB2, TIF35* and *YEF3B*) are downregulated after osmotic shock, leading to cell growth arrest (Rep et al. 2000). Furthermore, the transcription of the regulatory proteins RAP1 and FHL1 are also downregulated (Liu 2011). These TFs are involved in the regulation of ribosome biogenesis and protein translation processes.

The cell employs mechanisms to ensure that the translational capacity is sufficient to stimulate the production of the proteins needed for the adaptive responses.

2.14.3.9 Ubiquitin-mediated proteasome degradation pathway (UPP)

Environmental and chemical stress disrupt protein conformation, causing the proteins to unfold and aggregate (Goldberg 2003). Protein chaperones facilitate the refolding of unfolded proteins, however, acute or prolonged stresses may cause irreversible protein damage leading to an accumulation of unfolded proteins. Misfolded, damaged and aggregated proteins are toxic and are therefore degraded by the UPP as a means of maintaining normal cell function, including cell cycle regulation, metabolic adaptation and gene regulation (Varshavsky 1997; Glickman and Ciechanover 2002; Goldberg 2003; Wang et al. 2010). Directing the polyubiquitinated proteins to the 26S proteasome for degradation prevents adverse effects on cell integrity and viability (Varshavsky 1997; Glickman and Ciechanover 2002). The increased expression of genes involved in the UPP (*HUL5, UBC4, UBC6, UBP3, UBP6* and *UBP9*) assist in the degradation of denatured proteins (Boy-Marcotte et al. 1999; Yamamoto et al. 2008; Ismail et al. 2013). These degradation mechanisms are regulated by the TFs RPN4 and HSF1 with co-regulation by the TFs YAP1 and PDR1 (Liu 2011).

Several UPP genes (*ECM29, OTU1*, *PRE1, PRE3, PRE6, PRE7, PRE10, PUP3, RPN9, RPN12, RPT2, RPT3, RPT4* and *SHP1*) required for protein degradation are affected during HMF adaptation (Mannhaupt et al. 1999; Ma and Liu 2010b). These genes encode enzymes required for the degradation of damaged proteins to maintain cell viability and cellular functions during environmental, chemical and physiological stress. Transcription of these genes may be regulated by the TF RPN4 by binding to the proteasome-associated control element (PACE) (Mannhaupt et al. 1999). These conserved promoter elements are found in the promoter region of most UPP genes (Liu 2011). In addition, RPN4 is regulated by the 26S proteasome via a negative feedback control mechanism (Xie and Varshavsky 2001) (section 2.14.3.2).

Direct regulation of *RPN4* by HSF increases the proteasomal capacity, thus providing a mechanism to manage the stress associated with unfolded proteins. Moreover, genes in the ubiquitination pathway such as *UBI4, UBC4*, *UFD4* and *PIB1,* assist in the degradation of damaged proteins and are regulated by HSF (Seufert and Jentsch 1990; Simon et al. 1999). *UBI4* and *UBC4* encode a multi-ubiquitin and ubiquitin conjugation enzymes,

respectively, whereas *UFD4* and *PIB1* encode two ubiquitin ligases (Hahn et al. 2004). Thus, HSF plays a role in the co-ordinated regulation of UPP during acute stress involving permanent protein damage via direct and indirect mechanisms.

The integrated nature of the various stress responses indicate that tolerance to various stresses is dependent on complex co-ordinated networks that are interdependent on one another. *Saccharomyces cerevisiae* spp. are suitable for large-scale fermentations because of their tolerance to stress and their ethanol productivity under anaerobic conditions (Zhao and Bai 2009). The fermentation capacity and inhibitor resistance in natural strains can be higher than that of established industrial and commercial *S. cerevisiae* strains (Favaro et al. 2013). Since, resistance to stress conditions is strain-dependent (Ramos et al. 2013), multiple stress tolerant strains can be obtained from conventional and unconventional ecological niches (Favaro et al. 2013).

2.15 *SACCHAROMYCES CEREVISIAE* STRAIN IMPROVEMENT

To obtain a suitable *S. cerevisiae* host strain for the production of second-generation bioethanol, the diversity of natural strains can be further improved by using two possible strategies: obtaining natural multi-tolerant strains through classical genetics via the hybridisation of phenotypically resistant strains or by genetically engineering multi-tolerance into existing industrial strains.

To exploit *S. cerevisiae* as a CBP host, the organism also needs to be engineered to produce cellulases (for cellulose hydrolysis) as well as enzymes required for the fermentation of pentose sugars. Genetic manipulation of microorganisms may increase the metabolic burden of these strains. Furthermore, production of the proteins encoded by the engineered genes divert energy away from the general metabolism and place strain on the secretion pathway, which could induce stress (Görgens et al. 2001; Lynd et al. 2002; Van Rensburg et al. 2012; Tang et al. 2015). This may negatively affect bioethanol production.

2.15.1 Classical genetics

Classical genetics entails the generation of hybrid progeny that contains the complete genome of both parental strains, thus allowing for an increase in genetic diversity and providing a simplistic way to alter the genome of an organism. Hybrids are considered to be better adapted to intermediate or fluctuating situations by acquiring physiological

traits of both parental strains (González et al. 2006; Belloch et al. 2008). The outcome, however, is unpredictable and requires screening of several hybrids to obtain strains exhibiting the enhanced properties of interest. Various techniques can be used to combine two strains through mating, including classic mating, protoplast fusion and spore-to-spore mating.

Classical mating is the deliberate breeding between haploid strains of opposite mating types that allow for the generation of genetic diversity due to the hybridisation of two genetically diverse strains. Genetic recombination may occur, increasing the genetic diversity and the possibility of generating unique phenotypes.

Protoplast fusion involves the genomic hybridisation of two different organisms without the need for mating to occur (Scheinbach 1983), thus broadening the range of strains that can be mated. This allows for both inter- and intraspecies mating, including the mating of polyploid and aneuploid strains. Furthermore, protoplast fusion permits the incorporation of cytoplasmic elements into the generated hybrids, including mitochondria and plasmids (Scheinbach 1983). A major drawback of protoplast fusion is the unstable nature of the progeny (Scheinbach 1983). In addition, hybrids generated by protoplast are considered to be GMOs because of the manipulation that is required to remove the cell wall, leading to artificial fusing and forced recombination of traits (Pérez-Través et al. 2012).

Spore-to-spore mating includes the hybridisation of individual spores generated after sporulation of diploid strains (Pérez-Través et al. 2012). Mating between spores from different parental strains increases genetic diversity, but the hybrids generated could be deficient in some of the industrially relevant characteristics present in the parental strains because of chromosomal segregation during meiosis and spore generation preceding hybridisation (Gimeno-Alcañiz and Matallana 2001; Caridi et al. 2002; Marullo et al. 2004).

Several hybridisation scenarios are possible during spore-to-spore mating: intertetrade mating occurs when spores of different asci and opposite mating types germinate and mate to produce heterothallic strains. Intratetrad mating entails the mating of spores with opposite mating types generated in single asci to produce heterozygous progeny. The homothallic nature of natural strains allows haplo-selfing (also referred to as rare-

mating) to occur, during which, mating-type switching allows the mating of genetically identical strains (Pöggeler 2001). Homozygous diploid strains that are produced, permit recessive traits to be displayed, increasing the possibility of producing novel phenotypes. This process favours genetic variation within a single cell through spontaneous mutations, genome renewal and mitotic crossover (Rainieri et al. 2003; Peter et al. 2018). Genome renewal has been implicated as one of the major drivers of genetic variation in homothallic cultures (Steensels et al. 2014; Guillamón and Barrio 2017).

2.16 MOLECULAR TECHNIQUES TO DETERMINE ADAPTATION TO ENVIRONMENTAL CONDITIONS

Several transcriptomic studies have been performed to elucidate the molecular mechanisms that confer tolerance to environmental conditions. These studies report differential expression of several genes, including genes involved in central metabolic processes, structural processes, transport, stress responses, enzyme activity, oxidoreductase activity, protein activity, intracellular homeostasis, transcriptional regulation, ubiquitin-dependent protein catabolic process, MAPK signalling pathways, cell cycle and programmed cell death (Nookaew et al. 2012; Bajwa et al. 2013; Chen et al. 2016; Thompson et al. 2016; Dong et al. 2017). This confirms that stress tolerance is a polygenic trait that requires the co-ordinated expression of several genes to maintain cell viability and ethanol productivity.

Polygenic traits such as inhibitor and temperature tolerance thus require integrated approaches to understand their functional and molecular mechanisms. Modern molecular genetic platforms rely on computational analysis to analyse biological data. Large data sets are generated that require methodical evaluation and can only infer mechanisms responsible for a specific biological process. Functional experiments should be conducted to confirm any conclusions drawn from the data. “Omic” technologies (also referred to as high-dimensional biology) employs a universal approach of the molecular composition of an organism, and detect genes (genomics), mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics) in a non-targeted and non-biased manner (Horgan and Kenny 2011)

Next-generation sequencing (NGS) methods are based on the fragmentation of genomic DNA, which are sequenced and aligned to a reference sequence (Manzoni et al. 2018). Genomic sequencing allows the identification of novel genes as well as genes that may

not be expressed, and therefore does not allow for the detection of mechanisms involved in a specific process during a specific experimental condition. Furthermore, it only allows for the detection of coding regions and the role of non-coding regions cannot be determined (Manzoni et al. 2018).

Quantitative trait loci (QTL) mapping allows the localisation of chromosomal areas that affect the variation of quantitative traits in a population and is used to infer the relationship between a genotype and a phenotype (Zheng 2013). QTL mapping provides reliable statistical power, but low resolution for detecting a QTL. It detects all the genes associated with a phenotype, but not the specific SNPs, as it maps to a large region that may contain several genes (Barton and Keightley 2002; Cubillos et al. 2011; Swinnen et al. 2012).

Microarrays measure differences in the DNA and/or RNA sequence between strains with a set of pre-defined oligonucleotide probes that are spread across the full genome/transcriptome or enriched around areas of interest using comparative hybridisation (Horgan and Kenny 2011; Manzoni et al. 2018). Prior knowledge of the genome is required, which affects the effectiveness of this technology (Hurd and Nelson 2009) and only genes/transcripts for which probes are available are detected, which excludes the identification of unknown and unidentified genes (Hurd and Nelson 2009; Manzoni et al. 2018). In addition, cross-hybridisation is required that limits analysis to non-repetitive sections and complicates the analysis of associated genes, alternatively spliced transcripts, allelic gene variants and SNPs. Furthermore, low-abundance sequences are difficult to detect (Hurd and Nelson 2009).

Transcriptomics provide access to the full complement of RNA transcripts in a cell, including coding and non-coding RNAs (Manzoni et al. 2018). It reflects the genes that are actively expressed at a specific time point and is used to determine the cellular response during a specific environmental condition (Marioni et al. 2008; Horgan and Kenny 2011). Analysis of the mRNA profile provides insight into gene expression, including the presence/absence and quantity of a transcript, alternative/differential splicing and quantitative assessment of genotype influence on gene expression. This information is essential to understand the dynamics of cellular metabolism (Marioni et al. 2008; Manzoni et al. 2018).

RNA-sequencing (RNA-seq) permits the sequencing and quantifying of dynamic transcriptomes at high resolution, independent of transcript size, without preconceptions or prior knowledge of the genomes they are derived from (Marguerat and Bähler 2010). RNA-seq data is quantitative, accurate, sensitive and reproducible and can be used to determine RNA expression levels (Marioni et al. 2008; Marguerat and Bähler 2010). It allows detection of the precise location of transcript boundaries as well as connections between multiple exons, and provides data on whether and how exons are connected. In addition, RNA-seq can also detect sequence variations (for example, SNPs) in the transcribed regions (Marioni et al. 2008). However, RNA-seq is not without challenges: advanced computing resources are required to map the huge numbers of reads within a reasonable time and it has a relatively high error rate when detecting SNPs to determine allele-specific expression, thus higher sequencing depths are required. Furthermore, sequences that are rearranged or contain post-transcriptional modifications, cannot be directly mapped to the reference genome (Marguerat and Bähler 2010).

Proteomics is used to determine information flow within an organism through protein pathways and networks. It can be used to understand the functional relevance of proteins as the proteome is an active reflection of both genes and the specific environment (Horgan and Kenny 2011). Proteomics have a higher degree of complexity than genomics due to the complex mRNA-amino acid translation code and the large amount of conformations and modifications (phosphorylation, glycosylation and lipidation) that is required to produce a functional protein (Horgan and Kenny 2011; Manzoni et al. 2018). In addition, alternative splicing produces multiple isoforms of the same protein (Manzoni et al. 2018). Moreover, data analysis cannot be performed at the “omics” scale due to incomplete and/or inaccurate protein sequence databases and technical difficulties associated with mass-spectrometry bias (Horgan and Kenny 2011; Manzoni et al. 2018).

Metabolomics is the study of the global metabolite profiles in a cell during a specific environmental condition (Horgan and Kenny 2011). The metabolome is the ultimate product of gene transcription and thus nearest to the phenotype of the organism. It is diverse and contains many different biological molecules, increasing the physical and chemical complexity compared to the other “-omes” (Horgan and Kenny 2011). Metabolites are products of biochemical activity and are therefore easier to associate

with a phenotype, but the vast number of reactions (>1800) and metabolites (>1450) involved complicates analysis (Patti et al. 2012; Ramirez-Gaona et al. 2017).

Genome-wide association studies (GWASs) is an observational study of a genome-wide set of genetic variants in different individuals to ascertain whether a variant is related with a specific phenotype (Visscher et al. 2017; Manzoni et al. 2018). It provides a list of SNPs that frequently associate with a trait, but not the specific variants or genes responsible for the association, neither their function (Visscher et al. 2017; Manzoni et al. 2018). Potential biological processes associated with a trait can be determined (Visscher et al. 2017; Manzoni et al. 2018).

Saccharomyces cerevisiae robustness is influenced by both the genetic and phenotypic characteristics displayed by the relevant strain. Phenotypic diversity is more pronounced in natural strains and has been shown to be influenced by the environment as well as the geographical location that the organism occupies. Since natural strains adapt to their environment through both genetic and phenotypic mechanisms, organisms from diverse environments and geographies should display a diverse range of phenotypes. Genetic diversity is also influenced by the environment and can be enhanced through hybridisation. Spore-to-spore mating especially between homothallic strains, allow for maximum genetic diversification through genome renewal via chromosomal rearrangement and genetic recombination. Artificial hybridisation through spore-to-spore mating could therefore potentially increase the genomic diversity and hence the phenotypic diversity of natural strains. Gene expression regulation is fundamental in linking genotypes with phenotypes. RNA synthesis and maturation are tightly controlled and regulate the complex gene expression networks that drive biological processes. These networks need to be robust and malleable to allow rapid adaptation to environmental or genetic perturbations. Transcriptomic analysis is essential for deciphering the functionality of the genome, determining molecular constituents of cells and for understanding adaptation to physiological and environmental conditions (Marioni et al. 2008). In addition, RNAs transcribed from non-coding genome regions play fundamental roles in phenotype diversity. RNA-seq should thus allow the elucidation of the molecular mechanisms responsible for maintaining cellular viability in the presence of unfavourable environmental conditions.

This study combines classical and modern techniques to obtain *S. cerevisiae* strains for use in the production of cellulosic bioethanol. The natural diversity of *S. cerevisiae* strains were exploited to obtain strains that display unique phenotypes relevant in the biofuel industry. A modern molecular method (RNA-seq) was performed on two strains to identify the molecular mechanisms associated with temperature and inhibitor tolerance. In addition, spore-to-spore hybridisation was used to further enhance the phenotypic characteristics of the natural strains.

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CHAPTER 3

NATURAL *S. CEREVISIAE* STRAINS AS INDUSTRIAL HOST RESERVOIR

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CHAPTER 3: NATURAL *S. CEREVISIAE* STRAINS AS INDUSTRIAL HOST RESERVOIR

2.1 INTRODUCTION

Natural *Saccharomyces cerevisiae* strains display genome ploidy, copy number variations and genomic polymorphisms that allow genetic diversity (Rainieri et al. 2003; Aldana et al. 2007). In addition, phenotypic variation is observed in natural *S. cerevisiae* strains collected from various habitats (Kvitek et al. 2008; Camarasa et al. 2011). Habitat differences have been implicated as an important source of species divergence, including similar habitats in different geographic areas (Warringer et al. 2011). In addition, phenotypic selection of natural isolates has been successfully used in strain development in several industries, including winemaking and brewery industries (Belloch et al. 2008; Fleet 2008; Logan and Rabaey 2012).

Saccharomyces cerevisiae wine strains display a variety of strain-specific metabolic characteristics (Rainieri et al. 2003). Vineyard strains, for example, are able to survive the multi-stress conditions that are characteristic of this environment (Clowers et al. 2015), including limited nutrients, low pH, temperature fluctuations (20 – 45 °C), high osmolarity, exposure to weak acids and ethanol and the presence of fungicides (Mortimer 2000; Besnard et al. 2001; Favaro et al. 2013). Strains isolated from these environments are often more robust than industrial strains with regards to stress tolerance (Favaro et al. 2013) and display a higher phenotypic diversity (Kvitek et al. 2008). Furthermore, variation in fermentation capacity and ethanol tolerance exists among *S. cerevisiae* isolates (Stern 2014) and is likely to be prevalent in nature (Kvitek et al. 2008). Grape pomace therefore represents an ideal environment for isolating multi-tolerant yeast strains.

Tolerance to several environmental conditions is a prerequisite when developing a microbial host for cellulosic ethanol production. High temperatures are encountered during fermentation processes due to the exothermic fermentation process (Basso et al. 2011; Kumar et al. 2013). An increased fermentation temperature is also advantageous as cellulase enzymes function optimally at increased temperatures, allowing fermentation and saccharification of cellulose to be performed at the same temperature

(or a narrower temperature range), negating and/or minimising the need for cooling. In addition, *S. cerevisiae* shows an increase in metabolic activity at 35 °C in comparison to its optimal growth temperature of 30 °C (Belloch et al. 2008; Gonçalves et al. 2011; Wimalasena et al. 2014). Furthermore, tolerance to several compounds produced during the pretreatment and fermentation processes is required. In addition, high fermentable sugar concentrations are needed to maintain high ethanol titers for cost-effective product recovery (Haelssig et al. 2008). This requires high gravity suspensions, which increase the osmolarity of the feedstock and result in elevated amounts of toxic chemicals, lignin and cellulose. Since these compounds impair enzyme activity and cell growth (Caspeta et al. 2014), strains capable of withstanding these environments are preferred for the production of cellulosic ethanol.

South Africa has an established wine industry that dates back to 1659 and is the 7th biggest wine producer in the world (Floris-Samuels 2016). The wine regions of South Africa represents ~1.5 % of global grape vineyards and are spread over the Western, Northern and Eastern Cape, and KwaZulu-Natal. This large area includes a wide range of climates, geographies and soil types (Bonnardot et al. 2005). The majority of the vineyards are located in the Western Cape Province and can be divided into distinct wine regions (Fig. 3.1). The Atlantic and Indian oceans ensure a Mediterranean climate with dry heat and intense sunlight in summer with average daily temperatures from 23–40 °C (Bonnardot et al. 2005). Winters are cold and wet with an annual rainfall of approximately 1 500 mm in the Breede River Valley to 250 mm in the Klein Karoo (Robinson 2006).

The Constantia Valley, the oldest wine region has a lower average summer temperature (18–19 °C) and is characterised by moderate and wet winters, an annual rainfall of above 1 000 mm, and sandstone soil with high loam and granite concentrations (Robinson, 2006). The climate of the Stellenbosch district, the second oldest wine region, is influenced by the adjacent False Bay, lowering the average temperatures during summer to ~20 °C. This region consists of several unique soil types, including decomposed granite and sandy, alluvial loam (Robinson, 2006). The West Coast region (Durbanville, Olifants River, Piketberg and Swartland) is influenced by the Atlantic Ocean, and the soil consists of sandy topsoil with textured subsoil and is predisposed to periodical wetness. The South Coast region (Walker Bay, Elgin and the Overberg) located east of Cape Town, is

influenced by the Indian Ocean with a maritime climate and soil consisting of shale (Robinson, 2006). The Breede River Valley has a warm climate with lime-rich soils containing a high proportion of sand and shale (Stevenson 2005). The Robertson district, which is part of the Breede River Valley, has an average annual rainfall below 400 mm with average summer temperatures of ~22 °C, with calcium-rich alluvial soils.

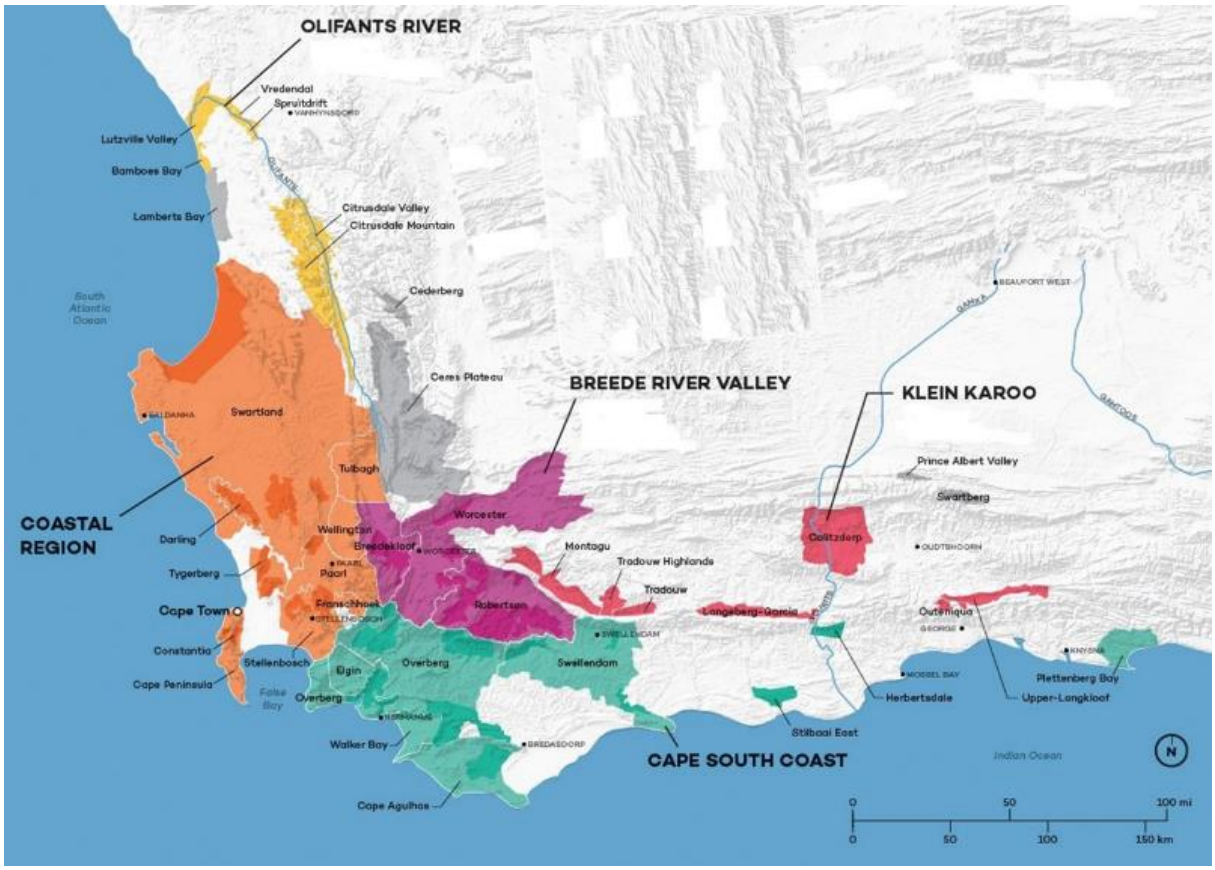

Figure 3.1: A Map of the Western Cape indicating the major wine regions, including the Coastal (West Coast), South Coast, Breede River Valley and Klein Karoo [adapted from Amazon.com Wine Folly South Africa https://www.amazon.com/Wine-Folly-South-Africa-Poster/dp/B0768KPTP9].

The geographical and climate diversity of the various vineyards in South Africa provides an ideal environment for generating phenotypic diversity. In this study, *S. cerevisiae* strains collected after the spontaneous fermentation of grapes from three areas in the Western Cape Winelands [Coastal (West Coast), Breede River Valley and Cape South Coast] were evaluated for their phenotypic characteristics and fermentation capacity.

2.2 MATERIALS AND METHODS

2.2.1 Strains

Natural indigenous *S. cerevisiae* strains from the Western Cape wine regions were obtained from the ARC-Infruitec Nietvoorbij. These strains were originally collected from vineyards in the West Coast, South Coast and Breede River Valley regions. Briefly, grapes were collected, allowed to ferment spontaneously and *S. cerevisiae* and non-*Saccharomyces* strains were isolated at the end of the fermentation process and evaluated and stored as part of the of the ARC-Infruitec Nietvoorbij yeast breeding and evaluation programme (Khan et al. 2000; Van Der Westhuizen et al. 2000a; Van der Westhuizen et al. 2000b). Fifty-six *S. cerevisiae* strains were randomly selected and propagated in YPD media (1 % yeast extract, 2 % peptone and 2 % glucose) and glycerol stocks (30 % v/v) were prepared. *S. cerevisiae* strain MH1000, a robust homothallic, industrial distillery strain, was used as a reference strain (Viktor et al. 2013). All subsequent experiments were performed in biological triplicates.

2.2.2 Phenotypic characterisation

Phenotypic characteristics were evaluated using plate assays, except for pH tolerance that was assessed in liquid medium. Serial dilutions (10^{-1}, 10^{-2} and 10^{-3}) of overnight cultures (i.e. stationary phase, grown in YPD medium and incubated at 30 °C) were prepared and spot inoculated onto the relevant agar plates, whereafter plates were incubated at 37 °C and monitored daily for 3 days. Temperature tolerance was investigated by spot inoculating onto YPD plates with incubation at different temperatures (26, 30, 37, 40, 42 and 45 °C). Ethanol, osmo- and inhibitor tolerance were assessed by spot inoculating onto YPD plates containing ethanol (10, 15 and 20 % v/v), NaCl (0.5, 1.0 and 1.5 mol/L) and a synthetic inhibitor cocktail (25, 50 and 75 %), respectively, with incubation at 30 °C. The synthetic inhibitor cocktail contained at least one representative of each of the major inhibitory compound groups found in various pretreated feedstocks, at concentrations previously reported as inhibitory for *S. cerevisiae* (Martín and Jönsson 2003; Jönsson and Martín 2016). The 25 % inhibitor cocktail contained: 0.88 g/L formic acid, 1.13 g/L acetic acid, 0.73 g/L furfural, 0.88 g/L HMF, 0.038 g/L cinnamic acid and 0.45 g/L coniferyl aldehyde (Martín and Jönsson 2003). Plates assessing growth in environments that contained volatile compounds (ethanol or inhibitory compounds) were sealed with parafilm in order to minimise

evaporation. These plates were monitored for growth on day 7 to determine whether evaporation played a role during the experimental procedure. pH tolerance was investigated by incubating the strains at 30 °C in 1 mol/L citrate buffered synthetic complete (SC) medium (1.7 g/L YNB, 5 g/L $(NH_4)_2SO_4$, 20 g/L glucose) with an initial pH ranging from pH 2-11. Cell growth was evaluated spectrophotometrically (A_{600nm}) for 3 days. The physiological tolerances were scored as follows: where multiple conditions for a single environment was tested (temperature, ethanol, pH and osmolarity), a score of 6 indicated growth after 3 days at the lowest inoculum concentration for all the conditions tested and a score of zero indicates no growth after 3 days for the concentrated inoculum for all the conditions tested. Where a single condition for an environment was tested (inhibitor), a score of six indicated growth after 1 day at the lowest inoculum concentration and a score of zero no growth after 3 days for the concentrated inoculum sample. Strains were evaluated for sporulation by inoculating overnight cultures onto sporulation plates (1 % potassium agar) with 7 days incubation at room temperature. Wet mounts were prepared and microscopically viewed for the formation of ascospores.

2.2.3 Glucose utilisation

Strains were screened for the ability to ferment glucose in a limited oxygen environment. Static fermentation experiments were performed at 30 °C as described by Favaro et al. (2013). Briefly, strains were grown overnight in must nutrient synthetic (MNS) minimal medium with 20 % (w/v) glucose (high glucose and limited nitrogen are indicative of the winemaking process) (Delfini and Formica 2001). Overnight cultures were inoculated (7.5×10^4 cells/mL) into serum bottles containing 100 mL MNS medium. Reaction vessels were sealed with rubber stoppers and each vessel was equipped with a syringe needle plugged with cotton wool for the removal of carbon dioxide (CO_2) produced during the fermentation reaction. Glucose utilisation was evaluated in the presence and absence of a synthetic 25 % lignocellulosic inhibitor cocktail. Growth was monitored daily by measuring weight loss in relation to CO_2 production. Results were reported (using a conversion factor of 2.118) as grams of glucose utilised. The conversion coefficient of 2.118 was empirically determined, considering some of the glucose is converted to glycerol (g/mol ratio ~0.072) with the remainder of the glucose converted to CO_2 (g/mol ratio ~2.048) (Delfini and Formica 2001). Fermentation reactions performed in serum

bottles were regarded as oxygen-limited due to the experimental set-up (limited headspace with a reaction volume of 91 % of the working volume; crimp sealed with a rubber stopper with no or low agitation (150 rpm). Fermentation experiments were conducted at pH 3.5, as an acidic environment is often associated with pretreated feedstocks, and a temperature of 30 °C as this is the optimal growth temperature of the strains.

2.2.4 Strain verification

Strains that performed well during the glucose utilisation experiments and displayed tolerant phenotypes were selected for further investigation. These strains were deposited in the Plant Protection Research Institute (PPRI) database in Pretoria, South Africa [part of the World Federation for Culture Collections (WFCC)]. The respective identification numbers for these strains are HR4: 21385; V3: 21381; YI13: 21378 and YI30: 21386. To confirm the authenticity of the selected strains, interdelta PCR using the delta 12-21 primer set (δ12; TCA ACA ATG GAA TCC CAA C and δ21; CAT CTT AAC ACC GTA TAT GA) (Legras and Karst 2003) and electrophoretic karyotyping (CHEF) was performed (Hoff 2012). For interdelta region PCR amplification, the 50 µl reaction volume contained 20 ng yeast DNA, 10x reaction buffer (Southern Cross Biotechnologies PTY (LTD), 25 mmol/L $MgCl_2$, 10 µmol/L of each oligonucleotide primer, 2.5 mmol/L of each dNTP and 0.5 U Super-Therm Taq polymerase (Southern Cross Biotechnologies Pty (Ltd). PCRs were performed with a BioRad cycler using the following programme: 4 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 48 °C and 90 s at 72 °C, and a final elongation step of 10 min at 72 °C. The selected strains were compared to a reference *S. cerevisiae* strain, CBS 1171, and the industrial *S. cerevisiae* strain, MH1000. Electrophoretic gels were run on a CHEF DRIII system (BioRad, USA) for 34 h at a constant voltage of 6 V/cm. The initial pulse duration was 30 s and the final pulse duration 215 s. A dendrogram was constructed by numerical analysis of CHEF karyotypes. Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA). Similarities between strains were calculated based on the Dice coefficient (Hoff 2012).

2.2.5 Aerobic growth characterisation

Shake flasks (1 L) containing 300 mL SC medium were inoculated (10 % v/v) with overnight cultures and grown for 24 hours on a rotary shaker at 200 rpm at 30 °C with sampling every 3 hours. Cell growth was evaluated spectrophotometrically (A_{600nm}).

Glucose utilisation and ethanol production were monitored by high-pressure liquid chromatography (HPLC), using a Surveyor Plus liquid chromatograph (Thermo Scientific) consisting of an LC pump, autosampler and refractive index detector. Compounds were separated on a Rezex RHM monosaccharide 7.8 x 300 mm column (00H0132-K0, Phenomenex) at 60 °C with 5 mmol/L H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min. Glucose and ethanol concentrations were also investigated in the presence of 10 % (v/v) ethanol and a synthetic 25 % inhibitor cocktail. Strains were grown for 3 days with sampling every 24 hours. Ethanol tolerance was evaluated to identify strains capable of ethanol production in the presence of an initial ethanol concentration of 10 %. Fermentation reactions performed in shake flasks were regarded as aerobic due to the experimental set-up (ample headspace with a reaction volume of 30 % of the working volume and agitation at 200 rpm).

2.2.6 Anaerobic ethanol production

Anaerobic ethanol production of the selected strains was assessed as described in sections 3.2.3 and 3.2.4 with minor modifications. Each fermentation vessel was equipped with a magnetic stirrer bar and two syringe needles; one plugged with cotton wool for the removal of CO_2 and one connected to a 2 ml syringe for sampling. The fermentation vessels were incubated on magnetic stirrers at 30 °C for 14 days. Fermentations were monitored daily by measuring cell growth spectrophotometrically (A_{600nm}) and ethanol and glucose concentrations using HPLC.

2.3 Results and discussion

Efficient and cost-effective conversion of cellulosic material to ethanol requires host strains that are ethanol, temperature, osmo, pH and inhibitor tolerant. These superior characteristics are required given the nature of cellulosic ethanol production i.e. ethanol as the final product, high optimal temperature of cellulase enzymes and inhibitory compounds formed during pretreatment (Taherzadeh and Karimi 2008; Della-Bianca and Gombert 2013; Liu et al. 2017). Nature harbours an enormous amount of diversity and the evaluation of natural strains with such superior characteristics for use as a microbial host is therefore essential.

The ability of natural *S. cerevisiae* strains to grow in different environments was evaluated and compared to a robust industrial distillery strain, *S. cerevisiae* MH1000. The

phenotypic assessments indicated that the natural strains display a variety of phenotypes as summarised in Fig. 3.2.

The pH and osmotolerance data were omitted as the strains did not differ in performance for these parameters. The pH at the end of the incubation period did not differ significantly from the initial pH. A maximum growth temperature of 42 °C was observed for the majority of the strains, with strain YI13 able to grow at 45 °C. Most of the strains displayed growth in the presence of 10 % (v/v) ethanol, but only a few strains were able to grow in the presence of 15 – 20 % (v/v) ethanol. A limited number of strains were capable of growth in the presence of a 25 % inhibitor cocktail, with none of the strains able to grow at the higher inhibitor cocktail concentrations. All strains were capable of growth at the pH range (pH 2-11) and salt concentrations (0.5 – 1.5 mol/L) tested (data not shown).

When assessing the sporulation ability of the strains, all strains were able to sporulate and were therefore most likely diploid. Glucose utilisation as an indicator of ethanol production was determined by measuring weight loss due to CO_2 production. The strains varied significantly in their ability to ferment glucose (Fig. 3.3). Overall, tolerant phenotypes were generally associated with the West Coast region, sensitive phenotypes were prevalent in the Breede River Valley isolates, and a higher frequency of strains with an increased fermentation capacity was observed in the Cape South Coast region.

The physiological (Fig. 3.2) and fermentation (Fig. 3.3) data were used to select four strains for further analysis. YI13 was able to grow in all the conditions tested (20 % ethanol, 25 % inhibitor cocktail and 45 °C) and displayed a moderate fermentation capacity, whereas V3 represents a mid-tolerant strain (growth at 15 % ethanol, 25 % inhibitor and 42 °C) with moderate fermentation capacity. HR4 was selected as a sensitive strain due to the ethanol- and temperature sensitivity (10 % and 40 °C) with low fermentation capacity, whereas YI30 displayed a varied phenotype with inhibitor tolerance (25 %), mild temperature tolerance (40 °C), sensitivity towards ethanol (10 %) and good fermentation capacity.

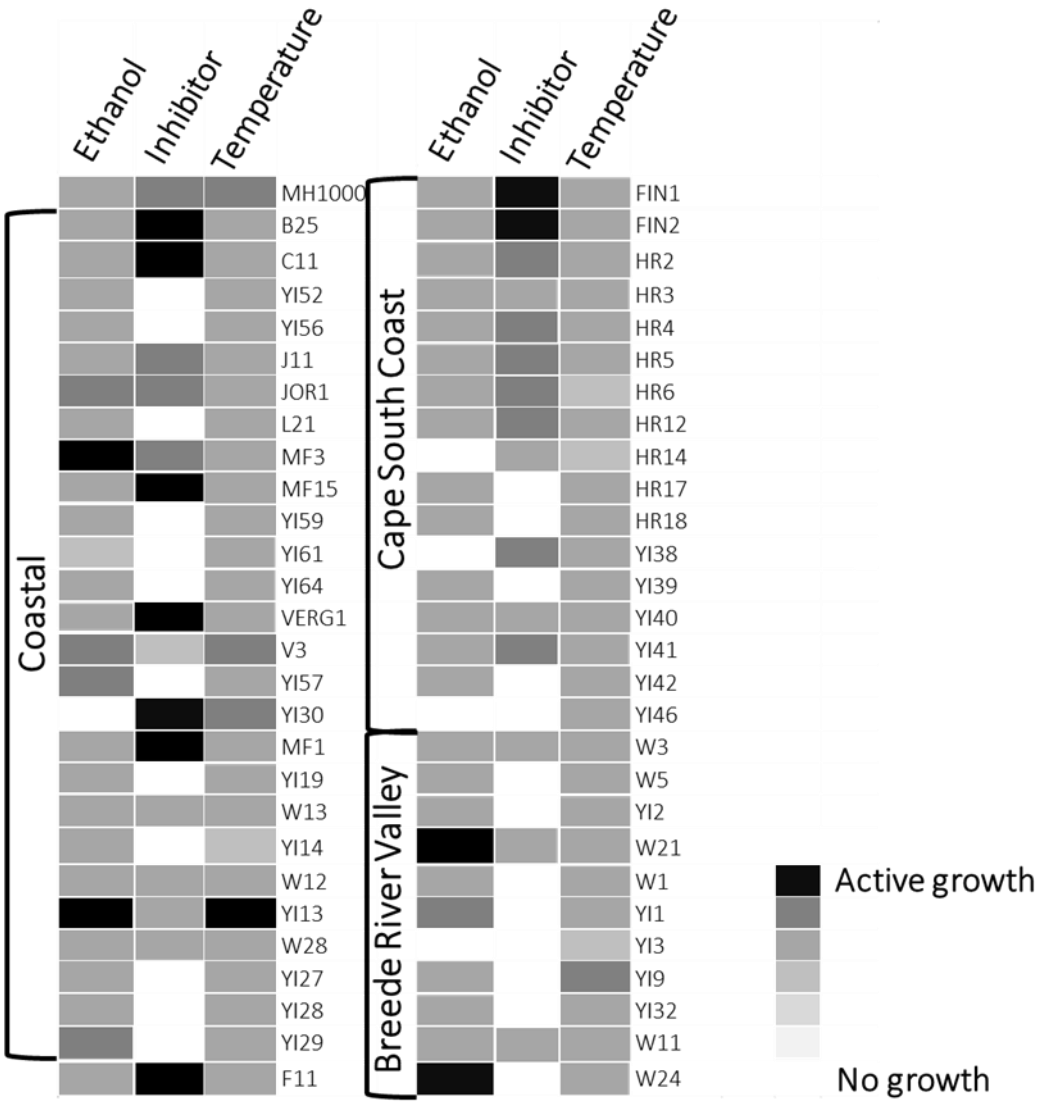

Figure 3.2: The viability of 56 natural strains in various environments was compared to the industrial strain, MH1000. Each row represents a different strain and each column a specific environment. Tolerance was scored as follows: where multiple conditions for a single environment was tested (temperature or ethanol); a score of 6 (black square) indicates growth after 3 days at the lowest inoculum concentration for the respective conditions tested and a score of zero (white square) indicates no growth after 3 days for the concentrated inoculum for the respective conditions tested. Where a single condition for an environment was tested (25 % inhibitor): a score of 6 indicates growth within 1 day at the lowest inoculum concentration and a score of zero no growth after 3 days for the concentrated inoculum [adapted from Kvitek et al. 2008].

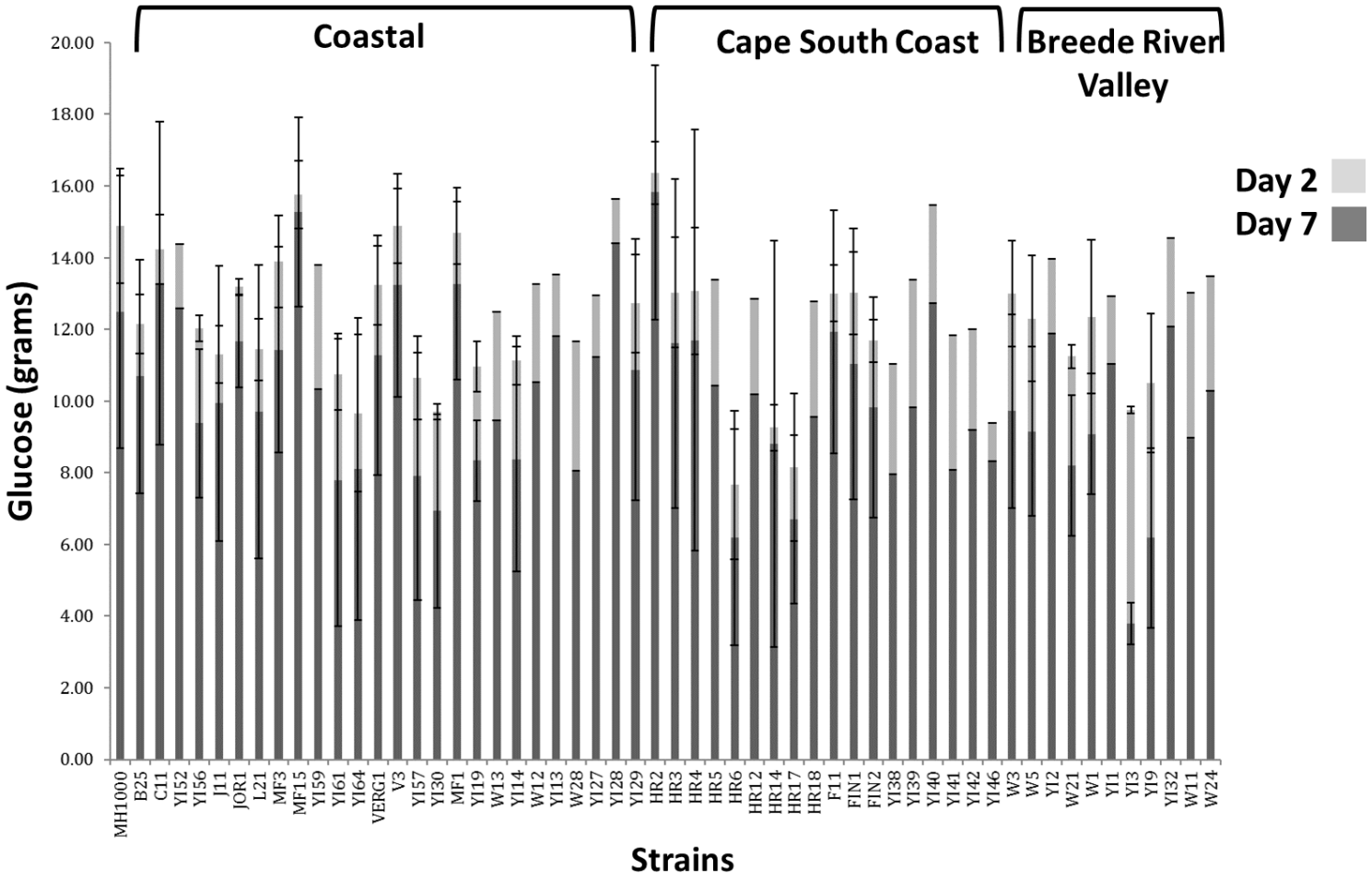

Figure 3.3: Static fermentations in MNS medium. Glucose was measured as weight loss via CO_2 production. The light gray bars indicate day 2 results, and the dark gray bars day 7 results. Data series and error bars represent the mean values and the standard error of biological triplicates. Data points where error bars seem to be omitted is due to low variation in biological triplicates.

CHEF karyotyping and interdelta PCR-based methods can be used to differentiate between *Saccharomyces* wine yeast strains (Hoff 2012). The phylogenetic analysis of the CHEF gel electrophoresis and interdelta PCR results revealed a close genetic relatedness of the selected natural strains to the *S. cerevisiae* type strain CBS 1171 (Fig. 3.4). The results indicated that the strains are distinctly different isolates, with the industrial strain MH1000 clustering separately from the natural strains (Fig. 3.4).

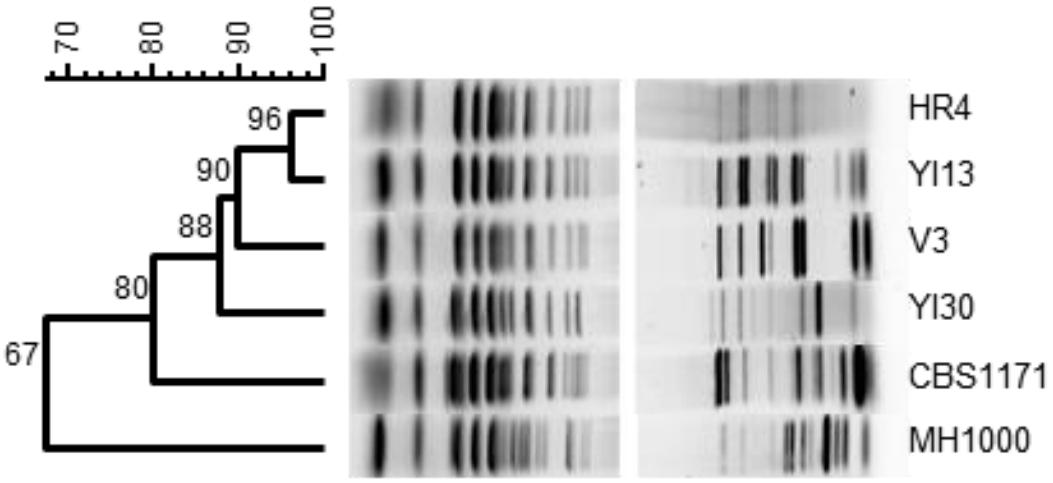

Figure 3.4: Dendrogram indicating the relatedness of selected strains. Clustering of the strains relative to the *S. cerevisiae* reference strain (CBS 1171) and the industrial *S. cerevisiae* strain MH1000 is indicated.

The growth, ethanol production, ethanol and inhibitor tolerance of the four selected strains were evaluated in aerobic (Fig. 3.5) and oxygen-limited (Fig. 3.6) environments. The majority of the strains were able to grow aerobically in the presence of 10 % (v/v) ethanol (Fig. 3.5 c), but none were able to ferment glucose in the presence of an initial ethanol concentration of 10 % (v/v) (Fig. 3.5 d). Except for strain YI30, all the strains were able to ferment glucose in the presence of a 25 % inhibitor cocktail. Strain HR4 was able to grow aerobically in the conditions tested (Fig. 3.5 c and 3.5 e) and produced the maximum amount of ethanol in the presence of a 25 % inhibitor cocktail (Fig. 3.5 f). This strain displayed the best growth during stress conditions, indicating a growth tolerance to unfavourable environmental conditions.

The inability of the strains to produce additional ethanol in the presence of an initial 10 % ethanol is due to the inhibitory effect of ethanol on yeast cells, as well as product inhibition. Ethanol inhibits cell division, reduces cell vitality and increases cell death. It

also has a negative effect on cell metabolism by denaturing proteins and reducing enzyme activity, whilst membrane fluidity and integrity are also affected. The metabolic flux of the cell is diverted from ethanol production to cell protection and the production of components required to combat the adverse effect of ethanol exposure (Stanley et al. 2010). Glucose was assimilated, but no ethanol was produced and limited growth was observed (Fig. 3.5 c), with the exception of strain HR4 that was able to grow well in the presence of 10 % ethanol. However, the growth tolerance of strain HR4 to ethanol was not observed during the plate assay (Fig. 3.2).

Growth was suppressed in most strains when exposed to an inhibitor cocktail, but these strains were still able to produce ethanol, with strain HR4 being the most effective at ethanol production from the available sugar. Strain YI30 displayed no fermentation capacity in the presence of the inhibitor cocktail during aerobic conditions. Inhibitors by definition have an inhibitory effect on cell viability and performance, affecting membrane solubility and integrity, reducing enzyme activity by denaturing proteins and disturbing the intracellular pH of the cell, as well as eliciting oxidative stress on the cell. These negative effects disturb cell metabolism, triggering a redistribution of metabolic flux toward repair and protective mechanisms and cell survival (Klinke et al. 2004; Van Maris et al. 2006; Almeida et al. 2007; Mills et al. 2009; Pienkos and Zhang 2009). The general decrease in growth observed in the majority of the strains during stress conditions reflects an increased need for ATP to manage the stress response mechanisms, thereby diverting energy away from biomass production (Viegas and Sá-Correia 1991; Verduyn et al. 1992; Holyoak et al. 1996).

Ethanol and inhibitor stress during oxygen-limited conditions (Fig. 3.6) had a similar effect on the growth of the strains, as none of the strains showed considerable differences (p-value < 0.05 for single-factor ANOVA) in the ability to grow in oxygen-limited conditions in the presence of either 10 % (v/v) ethanol or the inhibitor cocktail (Fig. 3.6 c and e).

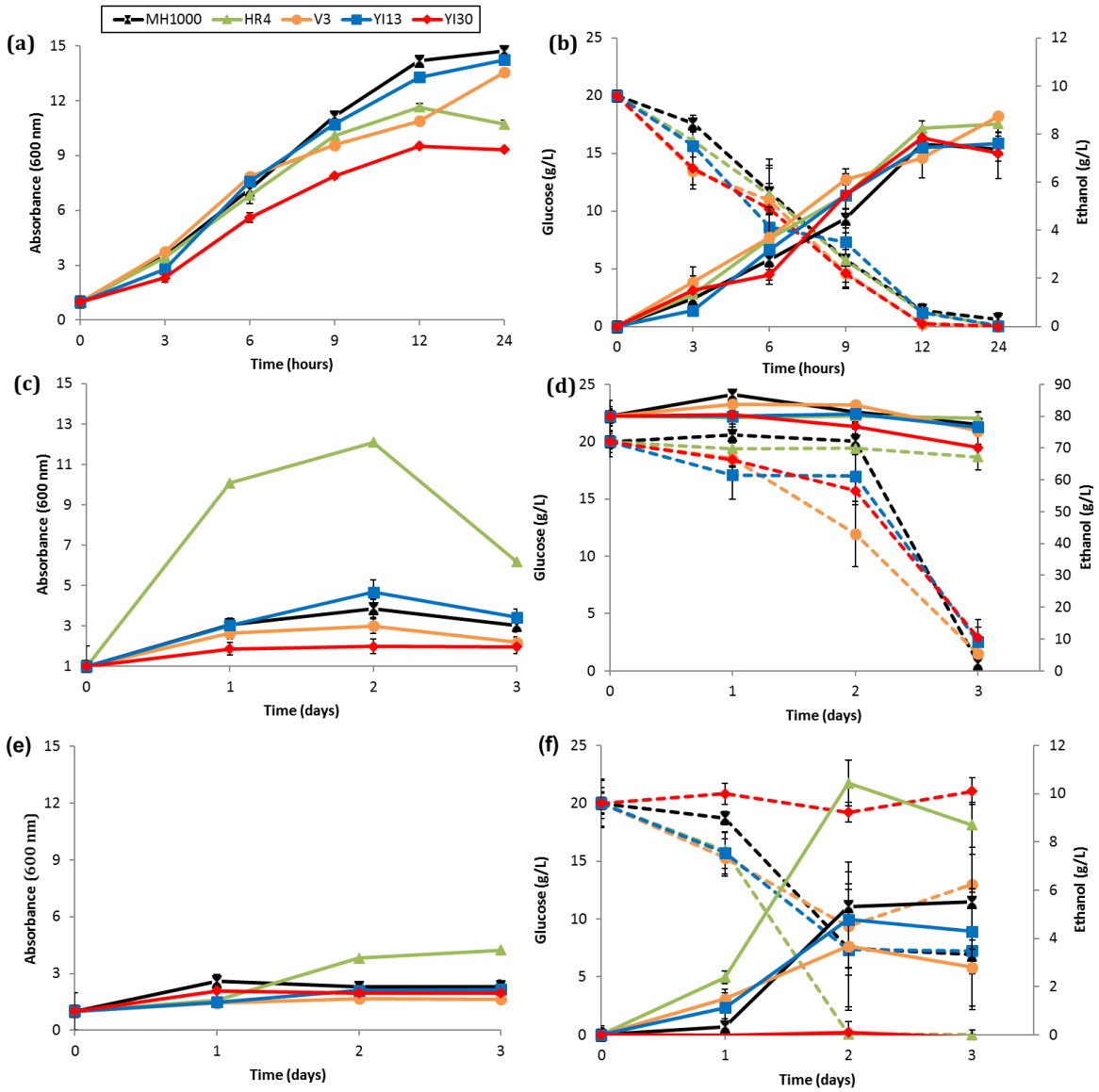

Figure 3.5: Aerobic fermentations of selected strains in SC medium. Cell growth (depicted in the left side panels) was measured spectrophotometrically at A600nm in (a) SC medium, (c) SC medium with 10 % ethanol, and (e) SC medium with 25 % inhibitor cocktail. Residual glucose levels and ethanol production (depicted in the right side panels) was monitored by HPLC. The broken lines indicate the residual glucose and the solid lines the ethanol produced during fermentations performed in (b) SC medium, (d) SC medium with 10 % ethanol, and (f) SC medium with 25 % inhibitor cocktail. Data series and error bars represent the mean values and the standard error of biological triplicates. Data points where error bars are not visible, are due to low variation between biological triplicates.

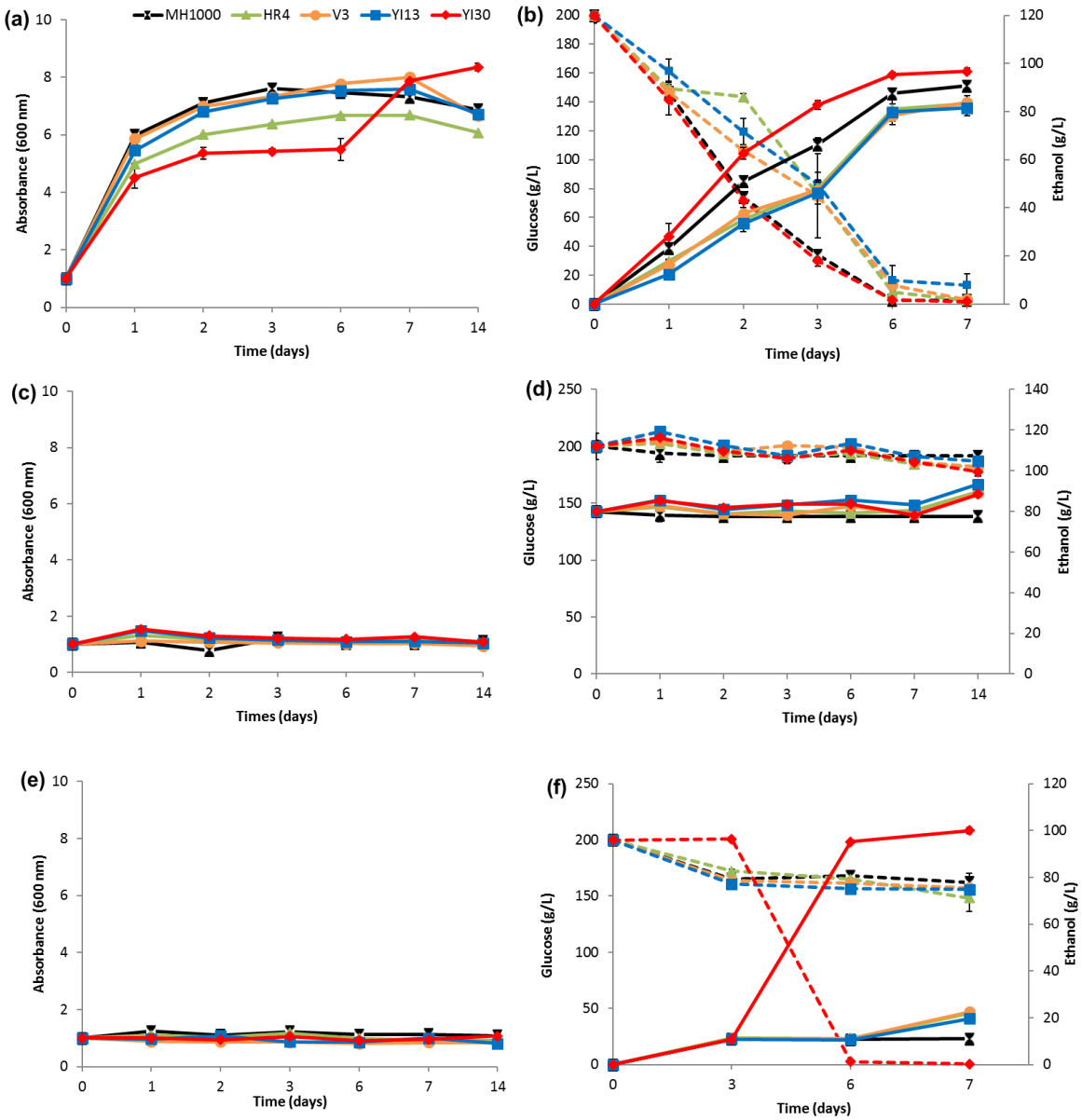

Figure 3.6: Oxygen-limited fermentations of selected strains in MNS medium. Cell growth (depicted in the left side panels) was measured spectrophotometrically at A600nm in (a) MNS medium, (c) MNS medium with 10 % ethanol and (e) MNS medium with 25 % inhibitor cocktail. Residual glucose levels and ethanol production (depicted in the right side panels) was monitored by HPLC. The broken lines indicate residual glucose, and the solid lines ethanol production. Fermentations performed in (b) MNS medium, (d) MNS medium with 10 % ethanol and (f) MNS medium with 25 % inhibitor cocktail. Data series and error bars represent the mean values and the standard error of biological triplicates. Data points where error bars are not visible, are due to low variation between biological triplicates.

The strains were unable to ferment in the presence of an initial 10 % (v/v) ethanol concentration (Fig. 3.6 d) and showed restricted growth in oxygen-limited conditions in the presence of the 25 % inhibitor cocktail (Fig. 3.6 e). In contrast to the aerobic fermentations, only strain YI30 was able to ferment glucose in the presence of the inhibitor cocktail (Fig. 3.6 f). This strain was unable to produce ethanol in the presence of an inhibitor cocktail when oxygen was available, but produced the maximum ethanol possible (theoretical yield based on glucose concentration) when oxygen availability was limited.

As with the aerobic fermentations, growth was reduced when the strains were exposed to ethanol and inhibitors respectively, but the reduction in growth was more pronounced. With the exception of strain YI30, ethanol production in the presence of the inhibitor cocktail was severely affected for all strains (Fig. 3.6 f). The increased sensitivity of the strains is due to the limited oxygen availability during the fermentation process. Though not specifically imposed, anaerobiosis occurred due to CO_2 production and the experimental set-up. This reduction in oxygen availability adds additional stress to the cell, as certain metabolic pathways do not function optimally during these conditions. Adjusting the metabolism to alleviate the stress associated with ethanol and inhibitor exposure is therefore limited, which could explain the decrease in glucose assimilation (Fig. 3.6 d and f).

Strain YI30 was able to assimilate glucose and produce ethanol in the presence of the inhibitor cocktail and absence of oxygen. Moreover, the presence of oxygen seems to inhibit the fermentation capacity of YI30. However, strain HR4 required oxygen to maintain fermentation capacity in the presence of inhibitors. Lignocellulosic inhibitor-derived tolerance in *S. cerevisiae* is attributed to various mechanisms and depend on the inhibitors present (Almeida et al. 2007) and the environmental conditions, including the availability of oxygen (Horváth et al. 2003). Horváth et al. (2003) reported that the detoxification mechanism for furfural differs during aerobic and anaerobic conditions. In aerobic conditions, furfural is converted to furoic acid and in the absence of oxygen, it is converted to furfuryl alcohol. *Saccharomyces cerevisiae* strains employ various mechanisms to survive in the presence of harmful compounds; these mechanisms are strain specific, which could explain the different inhibitor tolerances of the two *S. cerevisiae* strains, HR4 and YI30, under different environmental conditions.

The physiological characteristics of the selected strains were maintained throughout the study as well as after several generations of growth in rich glucose medium in the absence of the specific environmental pressure, indicating that the phenotypes of the natural strains were stable.

2.4 CONCLUSIONS

Evaluation of natural strains obtained from various environments produced strains with varied phenotypes that showed an association with the geographical area where they were collected. The natural strains did not differ with regard to pH and osmotolerance, with all strains displaying a wide range of pH and salt tolerance. We were able to identify a multi-tolerant strain, *S. cerevisiae* strain YI13, with the ability to grow at the maximum ethanol concentration and temperature tested (20 % and 45 °C respectively). *Saccharomyces cerevisiae* strain YI13 has potential as a microbial host in industrial processes that require fermentation at high temperatures.

Two strains, *S. cerevisiae* strain HR4 and YI30, were able to produce ethanol in the presence of the synthetic inhibitor cocktail (25 %) albeit during different fermentation conditions, i.e. aerobic and anaerobic. The ability of these strains to produce the maximum theoretical ethanol yield when exposed to an inhibitor cocktail, could be exploited for the production of cellulosic ethanol. These strains displayed similar temperature tolerances, but strain HR4 was more ethanol tolerant. The choice of microbial host will therefore depend on the industrial conditions of the fermentation process.

Although several strains displayed tolerances to different environmental conditions, no single strain was able to perform optimally at all the conditions tested. This indicates that obtaining a single ideal microbial host for application in all industrial processes is unlikely and that for each specific industrial process, a specific microbial host will need to be employed. Cellulosic bioethanol production industries will therefore need to generate a range of microbial hosts that will vary according to the specific conditions that are applicable, including the operating conditions and the specific feedstock and pretreatment method utilised. This is similar to the diversity that exists in various

brewing and winemaking industries, where each specific industry uses a specific inoculum depending on the specific beer or wine being produced.

Even though no single superior strain was identified, natural environments do harbour a range of strain diversity that allows for the isolation of strains with unique phenotypes (for example growth at 45 °C for *S. cerevisiae* strain YI13) and should be considered when strain diversity is required. However, aside from diversity in environmental conditions, differences in geographical locations also generate phenotypic diversity. Therefore, similar habitats in different geographical locations may allow for the identification of unique phenotypes that can be utilised in industrial and fundamental applications. Furthermore, mechanisms that allow environmental stress tolerance and adaptation could be elucidated by studying natural strains as a wide range of diversity are exhibited by these strains.

The strains identified during this study have potential application in the production of cellulosic bioethanol as well as understanding the mechanisms that allow inhibitor and temperature tolerance under such conditions. The economic feasibility of cellulosic ethanol production remains elusive due to the low oil price and the high production cost of cellulosic ethanol. The use of a robust microbial host strain in a CBP should be evaluated as it could potentially improve the production process and thus the economic feasibility of CBP, which is currently one of the most economical biotechnology for the production of bioethanol (Lynd et al. 2017; Wyman et al. 2017).

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CHAPTER 4

IMPROVEMENT OF NATURAL *S. CEREVISIAE* STRAINS THROUGH CLASSICAL GENETICS

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CHAPTER 4: IMPROVEMENT OF *S. CEREVISIAE* STRAINS THROUGH CLASSICAL GENETICS

4.1 INTRODUCTION

In nature, mitotic recombination is the predominant form of genetic recombination, suggesting that genetic drift plays a significant role in phenotype diversity (Warringer et al. 2011). Inter- and intraspecies hybridisation occurs naturally and could generate hybrid strains with superior or additional characteristics (Zörgö et al. 2012; Wimalasena et al. 2014). However, population studies have indicated that yeast mostly reproduce asexually and outcrossing is rare (Liti 2015). Sexual reproduction can occur within species, as well as between closely related species (Liti et al. 2006; Novo et al. 2009). The low occurrence of interspecies hybridisation is due to the impermeable nature of the asci cell wall, but natural hybrid cultures have been observed. Animals may assist in spreading *Saccharomyces sensu stricto* genes by ingesting yeast spores, whereafter the spores are hydrolysed by the enzymes in the digestive tract of the animal. The free spores are released in the faecal matter where they germinate and conjugate to generate inter- and intraspecies hybrids (Pulvirenti et al. 2002).

The diversity of microorganisms can be enhanced by the artificial generation of variants through several processes, including hybridisation of different strains (Steensels et al. 2014). Breeding between strains with similar characteristics generates greater variation in the progeny, suggesting a role for antagonistic alleles and epistatic interactions (Cubillos et al. 2011). Artificial inter- and intraspecies hybridisation amongst *Saccharomyces sensu stricto* yeast have been utilised to obtain yeasts for specific industrial applications (Benitez et al. 1996; Rainieri and Pretorius 2000; Gibbons and Rinker 2015). For example, crosses between different strains have been used in beer production to enhance the flavour, quality and stability of beer (Barnett 2007). The alloploidy displayed by natural strains allow multiple interspecies hybridisation events to occur, further enhancing genetic diversity (De Barros Lopes et al. 2002). Moreover, intraspecies hybridisation may occur, including intertetrade mating (mating of spores generated from different asci), intratetrade mating (mating with spores within the same asci) and haplo-selfing (mating of haploid cells with daughter cells after mating-type switching), increasing the possibility of generating genetic diverse progeny.

Sexual reproduction that allows for the generation of genetic diversity and therefore the phenotypic diversity of natural *S. cerevisiae* strains, can be exploited to generate hybrid *S. cerevisiae* strains with improved phenotypes. Various techniques exist that can be used to generate these hybrids, including mutagenesis, adaptive evolution, genome shuffling as well as classic mating techniques including protoplast fusion and breeding (Steensels et al. 2014). Hybrids of classic breeding programmes often have improved phenotypes relative to the parental strains (Meersman et al. 2015). In this study, we aimed to generate hybrids with improved phenotypes for lignocellulosic ethanol production through spore-to-spore mating of natural *S. cerevisiae* strains identified in Chapter 3.

4.2 MATERIALS AND METHODS

4.2.1 Strains

Natural *S. cerevisiae* strains (HR4, V3, YI13 and YI30) identified in Chapter 3 that displayed tolerance to different cellulosic ethanol stress phenotypes, were used for this study. *S. cerevisiae* strain MH1000, a robust, industrial distillery strain, was used as a reference strain (Viktor et al. 2013). All experiments were performed in biological triplicates.

4.2.2 Hybrid generation

Spore-to-spore mating was performed between the four *S. cerevisiae* strains. Briefly, strains were allowed to sporulate on sporulation agar for 7 days at room temperature. Asci were suspended in 2 mL sterile double distilled water (ddH_2O) and washed twice (centrifuged at 5000 rpm for 5 min and resuspended in 500 µL ddH_2O). Asci were resuspended in 150 µL lysis buffer (0.5 mg/mL Zymolase in 1 mol/L sorbitol) and incubated overnight at 30 °C. Spores were washed with 2 mL ddH_2O and pellets resuspended in 500 µL ddH_2O. The Singer MSM System 200 micromanipulator microscope (Singer Instruments, Somerset, England) was used to dissect the spores on YPD plates. Spores from different strains were allowed to mate and the diploids obtained were evaluated for the preferred characteristics.

4.2.3 Phenotypic characterisation

Phenotypic characterisation of the progeny strains was performed as described in Chapter 3. Phenotypes that were assessed, included temperature tolerance (26, 30, 37,

40, 42 and 45 °C), inhibitor tolerance (25 % synthetic inhibitor cocktail), pH tolerance (pH 2-11), and osmotolerance (0.5, 1.0, 1.5 and 2.0 mol/L NaCl). The physiological tolerances were quantified as discussed in Chapter 3.

4.2.4 Ethanol production

Ethanol production of the progeny was assessed during conditions simulating osmotolerance (20 % w/v glucose) and inhibitor tolerance (25 %). Fermentation reactions were performed as described in Chapter 3. Fermentations were monitored daily by measuring cell growth spectrophotometrically (A_{600nm}) and ethanol and glucose concentrations using HPLC. Ethanol yield (%) was determined from the slope of the curve of the fitted straight line obtained after plotting the ethanol produced (g/L) against the amount of glucose utilised (g/L). Ethanol productivity (g/L per h) was calculated as the maximum amount of ethanol (g/L) produced divided by the total time (hours) taken to produce the ethanol.

4.2.5 Strain verification

To assess the genomic profile of the generated progeny, interdelta PCR using the delta 12-21 primer set as well as electrophoretic karyotyping (CHEF) were performed (Hoff 2012) as described in Chapter 3. The generated progeny were compared to the parental strains.

4.3 RESULTS

Spore-to-spore mating of the selected strains produced viable progeny for all the combinations (HR4/YI13; HR4/YI30; V3/YI13; V3/YI30; YI13/YI30) except one (HR4/V3) combination. The inability of strains HR4 and V3 to generate progeny could be due to genetic incompatibilities, including lethal recessive mutations, thus producing non-viable progeny. The physiological characteristics of the generated strains (16 hybrids for each of the parental combinations) are summarised in Fig. 4.1; only progeny with phenotypes different from the parental strains are indicated.

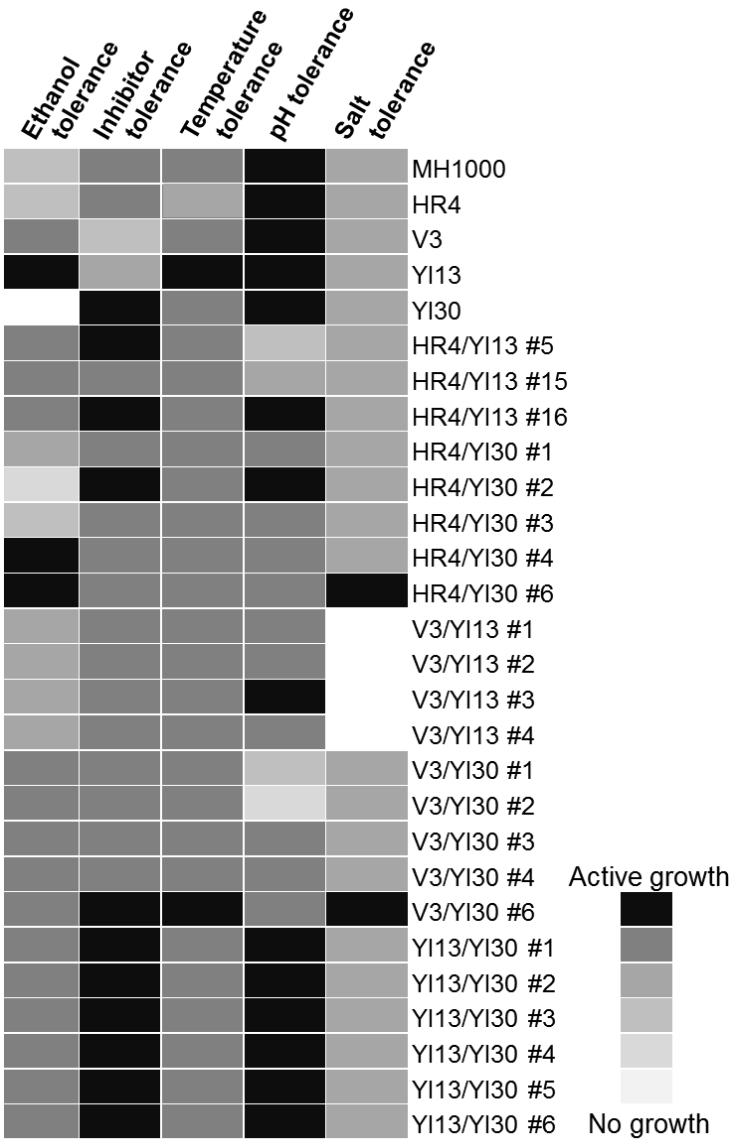

Figure 4.1: The phenotypic characteristics of the parental strains under different environmental conditions (ethanol, inhibitor, temperature, pH and salt) were compared to that of the progeny strains. Each row represents a different strain and each column a specific environment. The phenotype tolerance was scored as follows: where multiple conditions for a single environment were tested (temperature, ethanol, pH, or osmolarity), a score of 6 (black square) indicates growth after 3 days at the lowest inoculum concentration for the respective conditions. A score of zero (white square) indicates no growth after 3 days for the concentrated inoculum for the respective conditions. Where one concentration for an environment was tested (inhibitor): a score of 6 indicates growth within 1 day at the lowest inoculum concentration and a score of zero no growth after 3 days for the concentrated inoculum (adapted from Kvitek et al. 2008).

Hybrids generated from parental strains HR4 and YI13 presented phenotypes with both improved and diminished tolerances. Salt tolerance was unchanged, but sensitivity to pH increased except for strain HR4YI13#16, which maintained the high parental pH tolerance phenotype. An intermediate tolerance (higher than the sensitive parent, but less than the tolerant parent) was observed for the temperature and ethanol phenotypes. Strain HR4/YI13#5 and HR4/YI13#16 displayed an increased inhibitor tolerance in comparison to the parental strains.

HR4/YI30 progeny maintained the parental salt tolerance, except for strain HR4/YI30#6 that demonstrated an increased tolerance relative to both parental strains. The pH tolerance of the HR4/YI30 progeny decreased, except for strain HR4YI30#2, which revealed tolerance similar to the parental strains. The hybrids exhibited temperature tolerance equal to the more tolerant YI30 parental strain. Inhibitor tolerance did not differ from the parental strains, with progeny showing a similar inhibitor tolerance to either of the two parental strains. With regard to the ethanol tolerance, two strains (HR4/YI30#2 and #3) were more sensitive, and two strains (HR4/YI30#4 and #6) were more tolerant than the most tolerant parental strain, HR4.

Hybridisation between strains V3 and YI13 generated strains with unique phenotypic characteristics. Salt and ethanol tolerance for all the progeny decreased, whilst pH tolerance also decreased except for strain V3/YI13#3, which remained the same. Temperature tolerance for all the strains was similar to the least tolerant strain, V3, whereas inhibitor tolerance compared to the most tolerant strain, YI13.

Progeny produced by the V3 and YI30 crossing generated one strain (V3/YI30#6) that was more tolerant than the parental strains in both salt and temperature phenotypes. The remainder of the progeny was less salt tolerant, with similar temperature tolerance as the parental strains. V3/YI30#6 displayed a similar inhibitor tolerance phenotype as the most tolerant parent, with the remainder of the progeny presenting an intermediate inhibitor tolerance. All the strains showed a decrease in pH tolerance and an intermediate ethanol tolerance.

Outcrossing of strains YI13 and YI30 produced salt-sensitive progeny with a similar pH tolerance as the parental strains. Temperature tolerance was similar to the least tolerant strain (YI30), whereas inhibitor tolerance was similar to the most tolerant parental strain (YI30). An intermediate ethanol tolerance was observed.

In general, ten strains (43 %) of the produced progeny presented improved growth (growth at 10^{-3} dilution after 24 h) in the presence of the 25% synthetic inhibitor cocktail, compared to the parental strains (growth at 10^{-2} dilution after 48 h). Improved growth (growth at 10^{-3} dilution after 48 h) in an acidic (pH 2) environment was observed (for strains HR4/YI13#16, V3/YI13#3, HR4/YI30#2 and YI13/YI30#1-5) in comparison to the parental strains (growth at 10^{-3} dilution after 72 h). Contrary to the parental strains, strain V3/YI30#6 was able to grow at 45°C, strains HR4/YI30#4 and #6 were able to grow in the presence of 20% (v/v) ethanol, and strains V3/YI30#6 and HR4YI30#6 were able to grow in 2 M NaCl.

Progeny were evaluated for growth and ethanol production in a limited oxygen environment (Fig. 4.2 a-i). The HR4/YI13 and V3/YI13 strains demonstrated better growth than the parental strains (Fig. 4.2 a, and e), but less ethanol was produced and not all the glucose was assimilated by these strains (Fig. 4.2 b and f). The HR4/YI30 strains showed a decrease in growth (Fig. 4.2 c), but ethanol production (Fig. 4.2 d) did not differ when compared to the parental strains. Two of the YI13/YI30 strains (YI13/YI3#5 and #6) displayed enhanced growth (Fig. 4.2 i), but did not produce more ethanol (Fig. 4.2 j) than the parental strains. In addition, strains YI13/YI30#3 and #4 showed decreased growth with similar ethanol production profiles, and strains YI13/YI30#2 and #4 displayed similar growth and ethanol production profiles as the parental strains. The growth and ethanol production for the V3/YI30 hybrids did not differ significantly from that of the parental strains (Fig. 4.2 g and h).

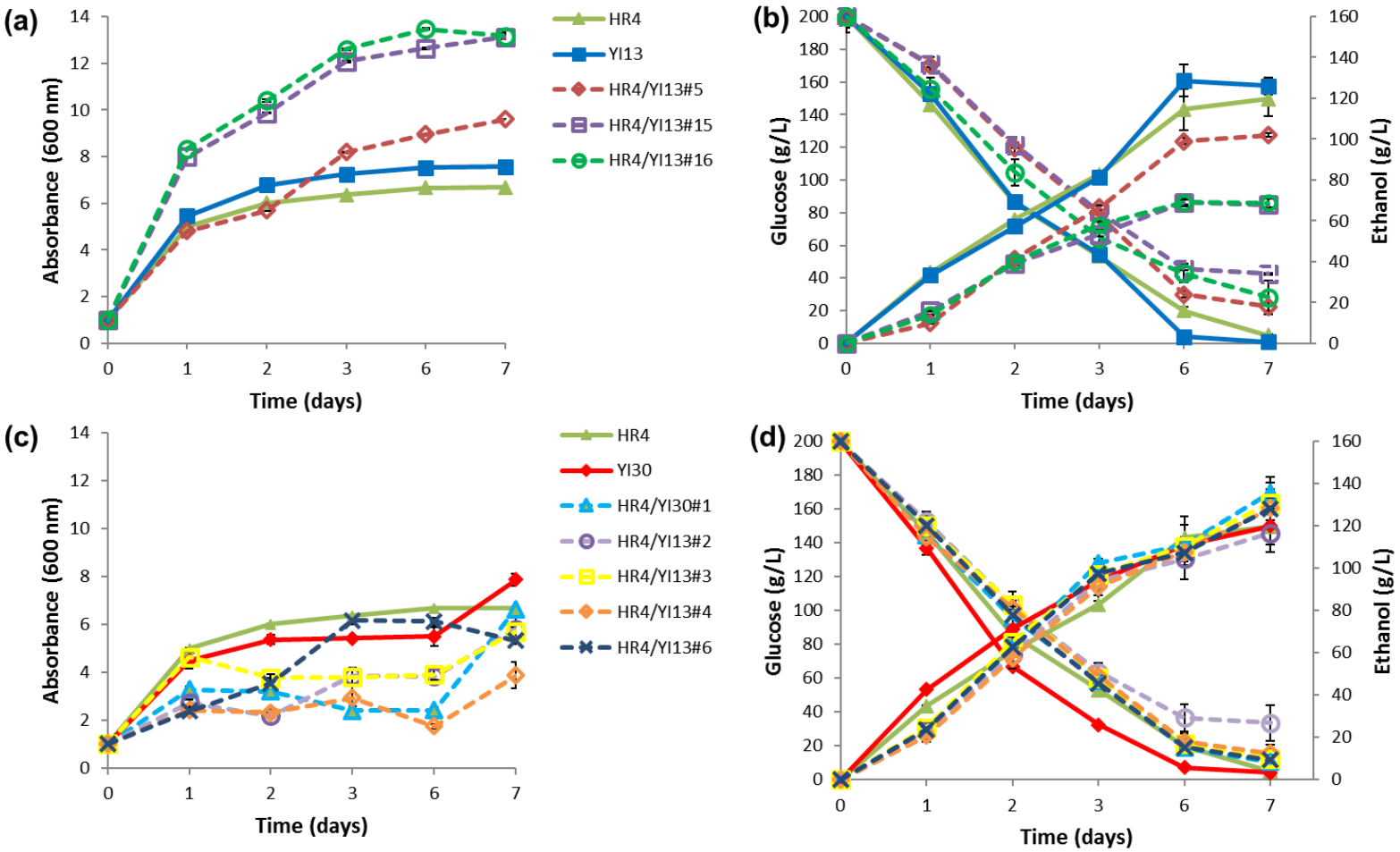

Figure 4.2 (a – d): Comparison of cell growth (a, c, e, and g) and ethanol production (b, d, f, and h) between parental (solid lines) and progeny (broken lines) strains in minimal medium.

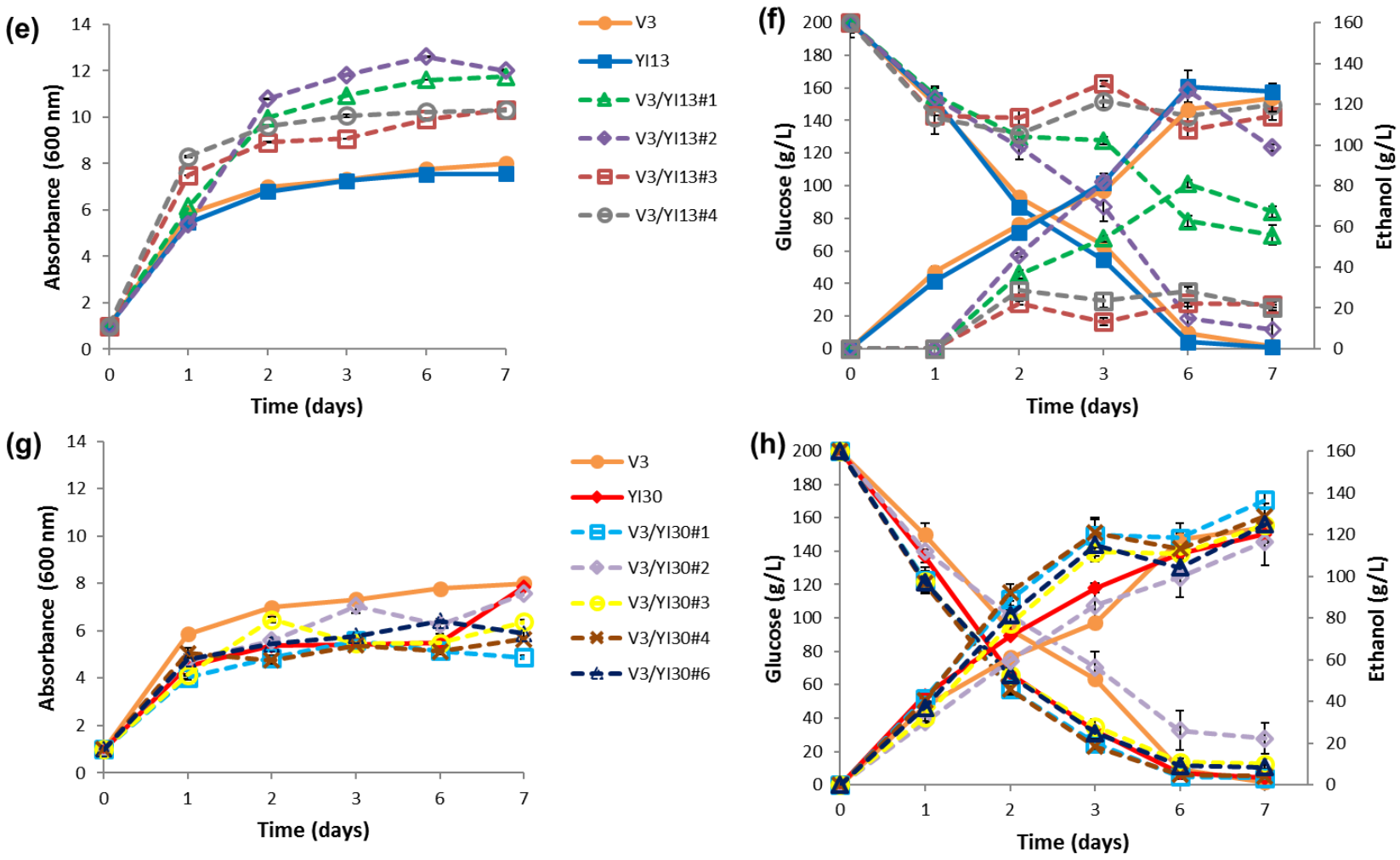

Figure 4.2 (e – h): Comparison of cell growth (a, c, e, and g) and ethanol production (b, d, f, and h) between parental (solid lines) and progeny (broken lines) strains in minimal medium.

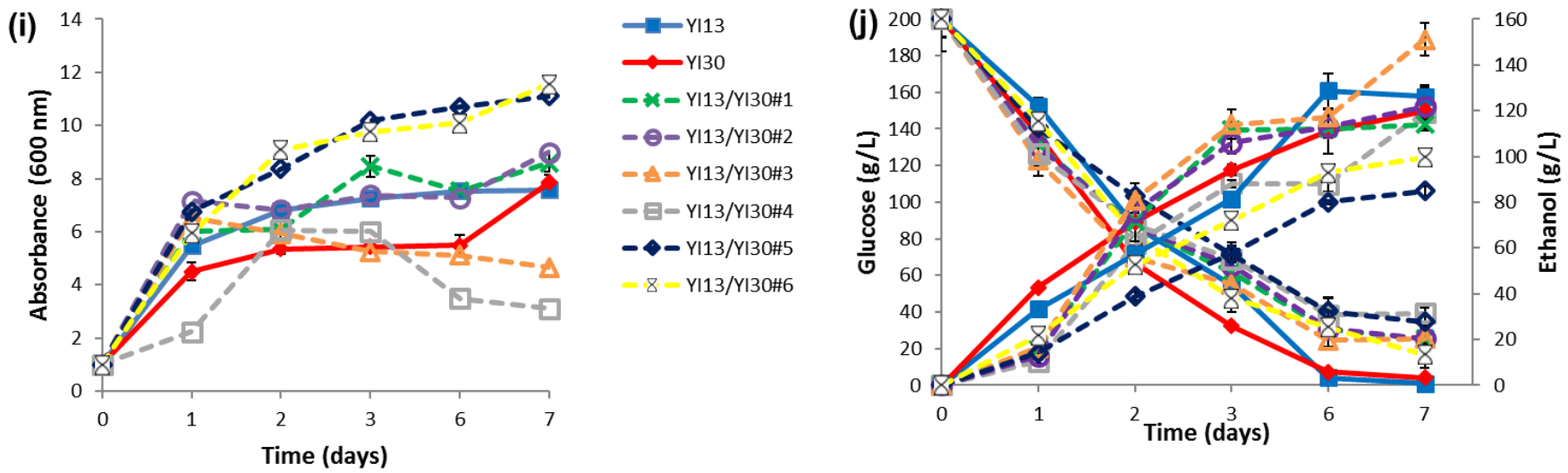

Figure 4.2 (i – j): Comparison of cell growth (i) and ethanol production (j) between parental strains YI13 and YI30 (solid lines) and progeny (broken lines) strains in minimal medium.

The ethanol yield (as a percentage of the maximum theoretical yield, where the theoretical maximum ethanol yield (100 %) is 0.51 g/L ethanol per 1 g of glucose) and productivity (g/L per h) for the generated progeny were determined and are represented in Fig. 4.3.

The progeny strains did not differ significantly in the total ethanol yield (Fig. 4.3), except for strains V3/YI30#6, YI13/YI30#2, #3 and #6, producing less than 60 % ethanol from the total sugar available. The productivity of the progeny (Fig. 4.3) varied, with strains V3/YI13#3 and #4 being less productive (> 0.3 g/L/h) than the parental strains (0.5 – 0.6 g/L/h). However, six strains (HR4/YI30#6, V3/YI13#2, V3/YI30#1, YI13/YI30#3 and #4), displayed increased productivity (< 0.7 g/L/h). The productivity of the parental and hybrid strains is, however, still below the productivity required for the cost-effective production of ethanol on an industrial scale (</= 1 g/L/h).

Ethanol production in the presence of a 25 % synthetic inhibitor cocktail was also evaluated. Only hybrids generated from the parental strains HR4 and YI30 namely (HR4/YI30#1 and HR4/YI30#3), were able to produce ethanol in the presence of a 25 % synthetic inhibitor cocktail (Fig. 4.4 b). Strain HR4/YI30#3 produced similar amounts of ethanol in the absence (Fig. 4.4 a) and presence (Fig. 4.4 b) of the inhibitor cocktail and displayed similar productivity (0.36 ± 0.0036 g/L/h) as the superior parental strain YI30 (0.35 ± 0.0058 g/L/h).

The physiological characteristics of the hybrid strains were maintained throughout the study as well as after several generations of growth in rich glucose medium in the absence of the specific environmental pressure, indicating that the phenotypes of the hybrid strains were stable.

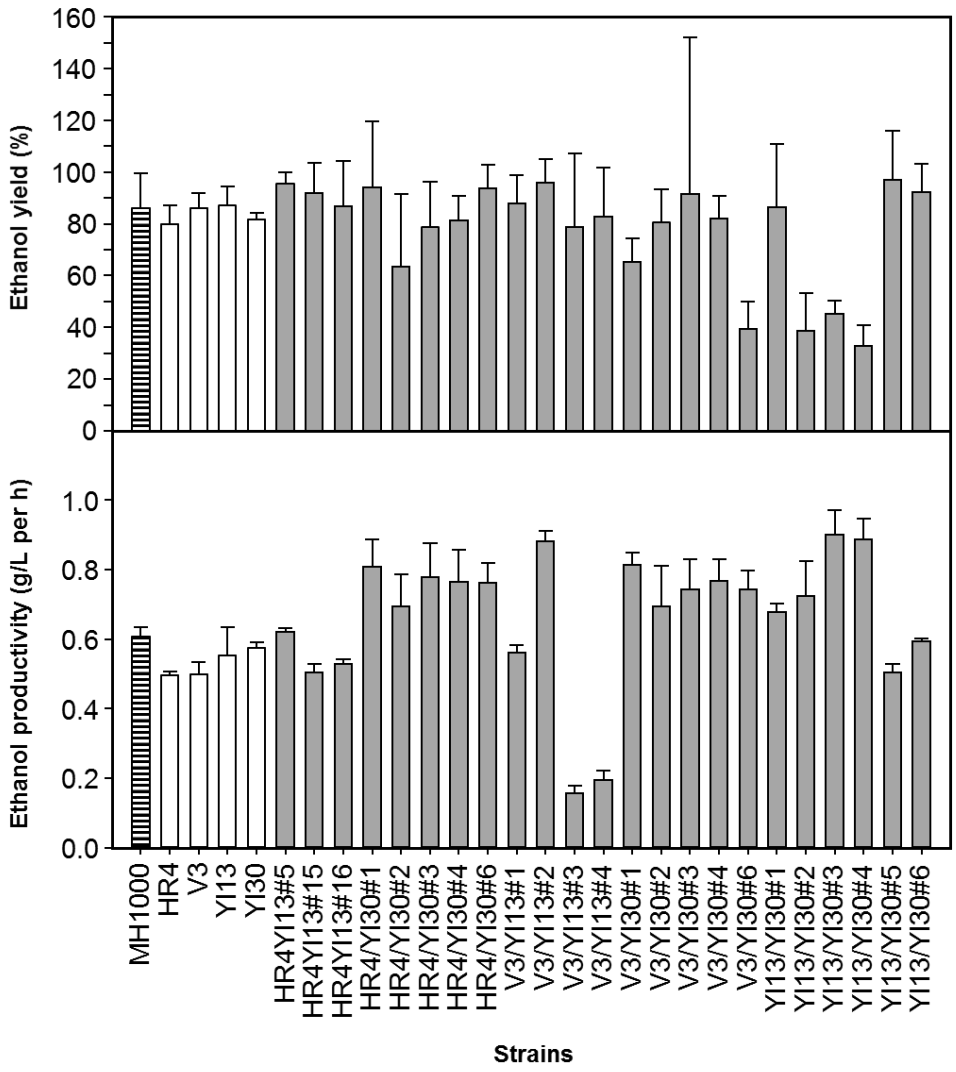

Figure 4.3: Comparison of ethanol production of parental strains (white bars) and generated progeny (gray bars) in minimal medium in a limited oxygen environment. The ethanol yield (%) was calculated from the slope of the curve of the fitted straight line obtained after plotting the ethanol produced (g/L) against the amount of glucose utilised (g/L). The maximum theoretical ethanol yield per gram of glucose is 0.51 g/L and represents 100 %. The ethanol productivity (g/L per h) was calculated as the maximum amount of ethanol (g/L) produced divided by the total time (h) required to produce the ethanol.

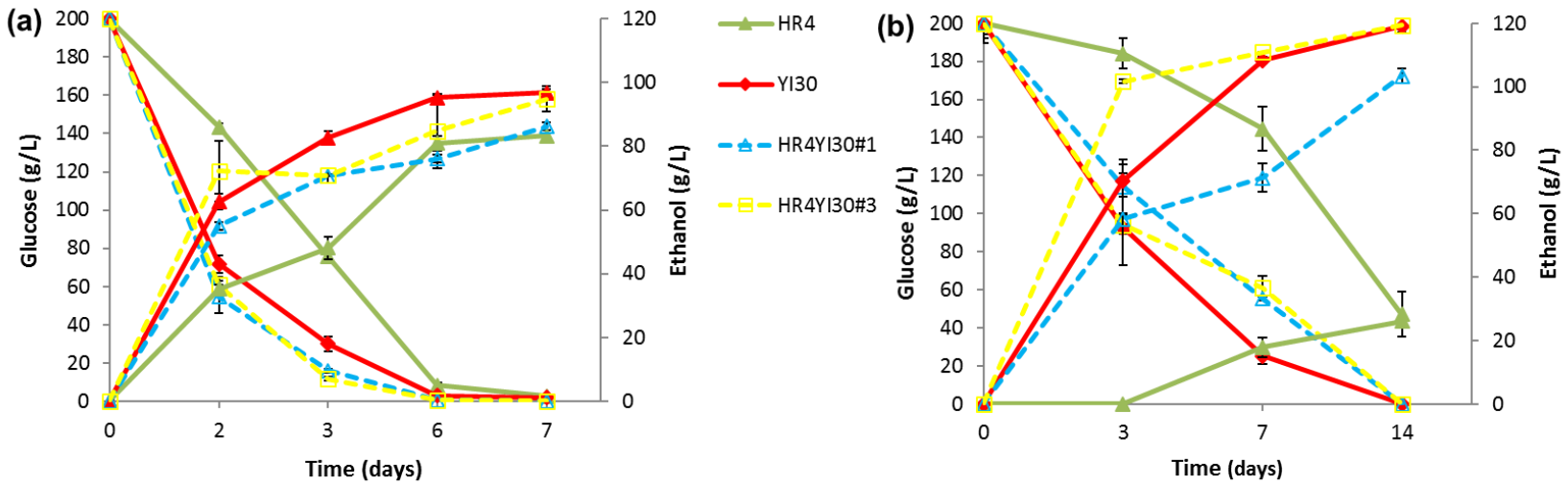

Figure 4.4: Comparison of fermentation ability of parental (solid lines) and progeny (broken lines) strains in the absence (a) and presence (b) of a 25 % synthetic inhibitor cocktail. Residual glucose and ethanol production are indicated. Data series and error bars represent the mean values and the standard error of biological triplicates. Data points where error bars are not visible are due to low variation in biological triplicates.

CHEF analysis was used to assess the karyotype profile of the generated progeny (Fig. 4.5 b - f). Similar karyotype profiles suggests that the chromosomal profile of the strain remained intact and that gross chromosomal rearrangement (whether due to recombination or segregation) did not occur, whereas unique karyotype profiles indicate that gross chromosomal rearrangement did take place. A combined karyotype (profile that displays all the bands of both parental strains) indicate the generation of true hybrid strains.

Mating between *S. cerevisiae* strains HR4 and YI13, V3 and YI13, and YI13 and YI30 produced strains with karyotypes unique from the parental strains and from one another (Fig. 4.5 b, c, and f). The HR4/YI30 mating generated three strains (HR4/YI30#1-3) with CHEF profiles similar to YI30, and two strains (HR4/YI30#4 and #6) (Fig. 4.5 d) with unique profiles. Two unique (V3/YI30#1 and #2) and three strains similar to YI30 (V3/YI30#3-4, and #6) (Fig. 4.5 e) were produced when *S. cerevisiae* strains V3 and YI30 were hybridised.

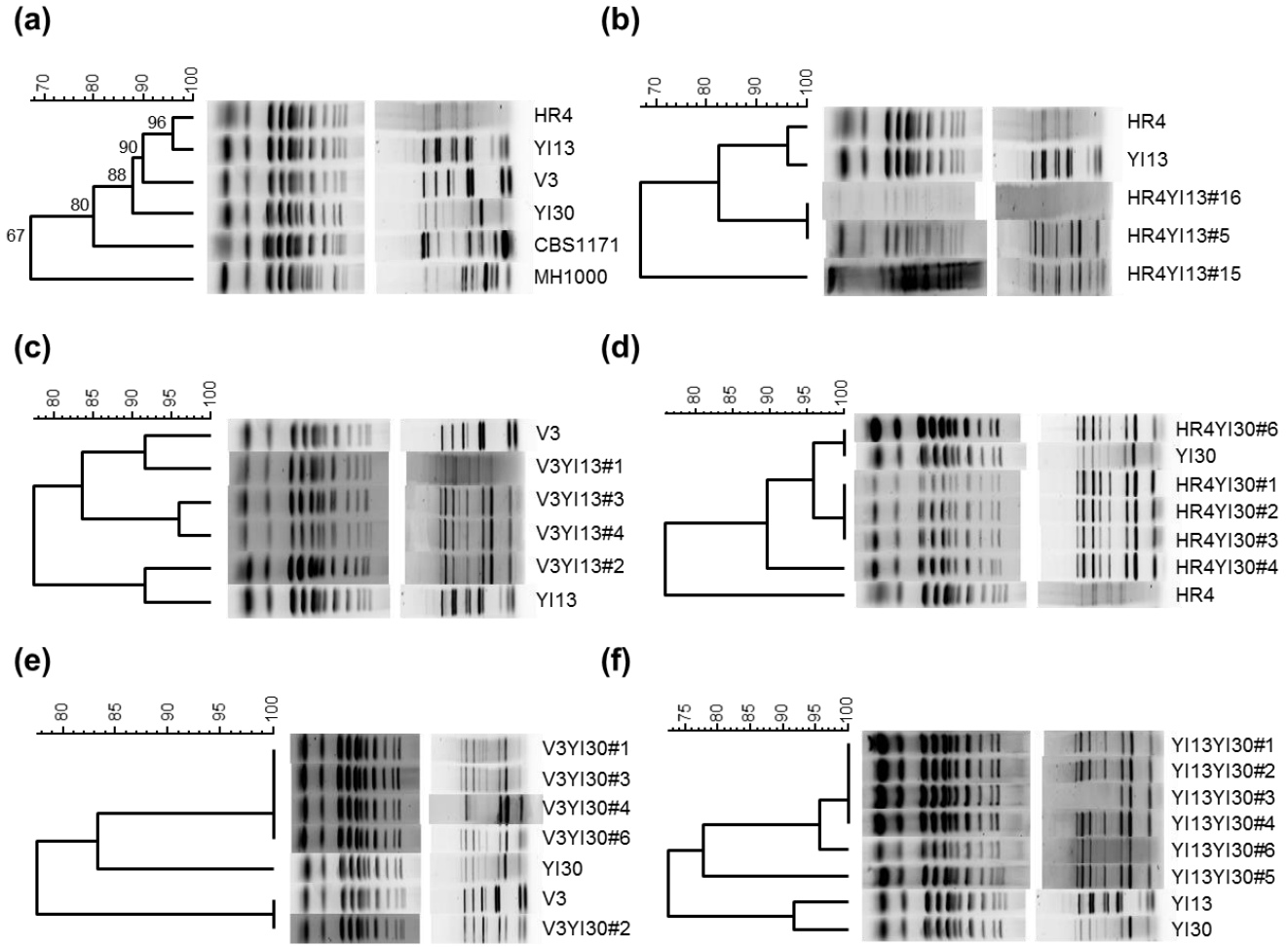

Figure 4.5: Dendrograms indicating the karyotype profile of the parental and hybrid strains. Clustering of the parental strains relative to the *S. cerevisiae* reference strain (CBS 1171) and the industrial *S. cerevisiae* strain MH1000 (a), as well as the progeny strains relative to the parental strains (b-f) are indicated.

4.4 DISCUSSION

Spore-to-spore mating is the targeted breeding of yeast strains providing an artificial method to increase yeast diversity. It may generate genetic diversity through changes in ploidy (i.e. gene dosage) as well as mitotic and meiotic recombination. Mitotic and meiotic recombination occurs mainly in telomeric regions and these regions have been associated with genes involved in environmental stress responses (Lopes et al. 2015). In addition, it has been suggested that polyploidy or aneuploidy is selected for, and may be induced during specific stress conditions, including stressed environments (Storchova 2014). This causes genomic instability that promotes chromosome missegregation, thus allowing chromosomal rearrangement and the development of variable karyotypes (Storchova 2014). Duplication of genetic material leads to an increased gene dosage and duplicated genes may evolve new functions that could lead to phenotypic variation (Storchova 2014; Matzke et al. 1999; Martinez et al 1995; Steensels et al. 2014). Ploidy changes due to hybridisation of strains that share environmentally stressed habitats, may also cause phenotypic variation in natural *S. cerevisiae* strains.

CHEF karyotyping is an effective method to identify hybrid strains as well as to detect gross chromosomal rearrangements. Hybrid strains display a karyotype profile that represents a combination of the parental karyotype profiles, whereas gross chromosomal rearrangements, (including changes in ploidy and recombination) is usually indicated by a unique karyotype with additional, missing or shifts in the banding profile (Oda and Ouchi 1990; Puig et al. 2000).

The fermentation capacity of the progeny was diverse, in general, mating with strain YI30 produced progeny with an increased productivity, whereas the opposite occurs when mating with strain YI13, and a decrease in productivity is observed. The karyotyping results indicated that hybridisation with YI13 promoted the generation of unique karyotypes, whereas mating with YI30 maintained the YI30 karyotype. This could explain the maintained and/or enhanced inhibitor tolerance displayed by the majority of the strains, as maintenance of the YI30 karyotype profile (with strain YI30 representing inhibitor tolerance) allowed for the persistence and/or enhancement of the inhibitor tolerant phenotype. It is suggested that chromosomal rearrangement could be the main

driver of diversity when YI13 is one of the parental strains, due to recombination between homologous chromosomes and repeated or paralogous sequences. However, genome renewal is suggested to be the main driver of diversity when YI30 is one of the parental strains due to the production of homozygous diploids after haplo-selfing, thus allowing for copy number variation (Puig et al. 2000).

The results of this study suggest that during artificial hybridisation, genetic diversity is strain-dependent, and enhanced and unique phenotypes can be attributed to both chromosomal rearrangements as well as genetic recombination. Aside from the myriad of parameters that influence genetic diversity, strain-dependent characteristics play an important role in generating genetic variation and therefore determine whether a specific phenotype will persist and could be improved, or will be lost through hybridisation.

4.5 CONCLUSION

In nature, the low incidence of outcrossing between strains limits the genetic diversity (Charlesworth and Wright 2001; Ruderfer et al. 2006) but outcrossing is an effective method to generate genetic diversity and thus phenotypic variation (Smukowski Heil et al. 2017). Artificial breeding strategies provide an alternative method to generate genetic diversity in *S. cerevisiae* strains and breeding strategies have been used to enhance industrial strains with complex traits, allowing for the generated hybrids that outperform the parental strains (Marullo et al. 2009; Meersman et al. 2015; Benjaphokee et al. 2012).

Although heterosis can be achieved, there is a limit in the degree of diversity attainable in a single organism. Robustness requires complex regulatory networks and increases the demand on the energy resources (Chapter 2 section 2.14). Although the regulatory networks used during adverse conditions generally rely on pre-existing pathways, the integration and increased flux through these pathways depend on increased energy generation while maintaining overall homeostasis. Due to the high energy demand of adaptation organisms are therefore able to display robustness against a limited number of environmental challenges, (De Visser et al. 2003; Maltsev et al. 2005).

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CHAPTER 5

TRANSCRIPTOMIC ANALYSIS OF GENE INDUCTION IN TEMPERATURE AND INHIBITOR TOLERANT NATURAL *S. CEREVISIAE* STRAINS

Chapter 5 is compiled in journal article format and is preparation for submission

Transcriptomic analysis of gene induction in temperature and inhibitor tolerant natural *S. cerevisiae* strains

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ABSTRACT

Saccharomyces cerevisiae strains have the ability to adapt to various adverse environmental conditions due to several molecular, cellular and biological processes. Two natural *S. cerevisiae* strains, YI13 and YI30, with superior tolerance to an increase in fermentation temperature and exposure to a synthetic inhibitor cocktail, respectively, were identified in a previous study. The transcriptional landscape of the two strains under relaxed and induced conditions were evaluated to elucidate the specific mechanisms responsible for temperature and inhibitor tolerance. The major molecular mechanism to maintain fermentation capacity in both the temperature and inhibitor tolerant strains related to maintaining redox homeostasis in response to an increase in oxidative stress. The rise in temperature increased the formation of reactive oxygen species that could affected several cellular mechanisms, causing irreversible protein damage, reducing oxygen availability and damaging membranes and DNA. The temperature tolerant strain YI13 responded by upregulating ribosome biogenesis and amino acid biosynthesis, thus increasing protein production and maintaining cell viability. The oxidative stress response was upregulated to combat oxidative stress and cell wall protein production was upregulated to maintain the integrity of the cell wall. The inhibitor tolerant YI30 strain responded to the inhibitor cocktail by detoxification and removal of the inhibitors. Detoxification could affect the redox potential of the cell, which could be restored by the upregulation of the oxidative stress response. In addition, the unfolded protein response was upregulated for correct folding of denatured proteins and genes involved in membrane stabilisation, were induced to combat the adverse effects the inhibitory compounds had on cellular membranes.

INTRODUCTION

The environmental stress response of *S. cerevisiae* allows the organism to maintain cell viability and ethanol productivity when exposed to adverse environments. This unique

phenotype along with its robustness when used in commercial ethanol production processes, make this organism the ideal host for the production of several value-added compounds from a variety of feedstocks, including harsh feedstocks obtained from industrial waste streams. Cellulosic bioethanol production using simultaneous saccharification and fermentation (SSF) or consolidated bioprocessing (CBP) processes would benefit from robust *S. cerevisiae* strains that can ferment at increased temperatures (Abdel-Banat et al. 2010) and in the presence of inhibitory compounds (Klinke et al. 2004).

Temperature tolerant host strains for industrial ethanol production are advantageous for various reasons. Heat is produced due to the metabolic activities of the host organism (Abdel-Banat et al. 2010), causing an increase in the environmental temperature. In regions where the average temperatures are high, cooling of fermentation systems are required to maintain processes at the desired temperatures (Kumar et al. 2013). However, higher temperatures are preferred as the optimal temperature for lignocellulose hydrolytic enzymes are between 45–50 °C (Grajek 1986; Lu et al. 2012). Temperature tolerant host organisms will be able to maintain cell viability and have enhanced metabolic activity at increased temperatures, which are compatible with optimal cellulase and hemicellulase activity, thus lowering the cooling required and concomitantly reducing operational costs (Yang et al. 2013; Saini et al. 2015). A reduction in cooling costs also enhances the sustainability of the process by reducing the water requirements. In addition, ethanol productivity is increased due to an enhanced enzyme activity at higher temperatures (Yang et al. 2013).

Inhibitor tolerant host strains are required for the production of cellulosic ethanol, as lignocellulosic material requires pretreatment prior to enzymatic hydrolysis and subsequent conversion of released sugars to bioethanol. The pretreatment process releases inhibitory components that affect the viability and ethanol productivity of the microbial host (Taherzadeh and Karimi 2008). An inhibitor tolerant host that is able to ferment in the presence of these inhibitory components will negate the need for detoxification steps and thus reduce the cost of producing cellulosic bioethanol.

Both temperature and inhibitor stress leads to the accumulation of reactive oxygen species (ROS) and unfolded proteins as well as membrane-, chromatin- and actin

damage (Lu et al. 2012). Furthermore, the fermentation process exposes yeast cells to several stresses, including high osmotic pressure, acidity, nutrient starvation and high alcohol concentrations.

The negative environmental habitat of yeasts elicits a general stress response, the common environmental response (CER) (Causton et al., 2001), as well as an environmental stress response (ESR) when adapting to a specific environment. Transcriptome analyses have identified ~499 CER genes and ~868 ESR genes that overlap by ~337 genes (Gasch et al., 2000). Genes induced in response to CER and ESR include carbohydrate metabolism, protein degradation and folding, ROS and genes with the stress response element (STRE) in their promoters. Repressed genes include those involved in translation, protein and tRNA synthesis and genes that encode cytoplasmic ribosomal proteins. In addition, many genes with unknown functions that respond to stress have also been identified (Gasch et al., 2000).

Transcriptomic analyses have been used to elucidate the complexity of gene expression regulation in *S. cerevisiae*. RNA-seq is a powerful tool for transcriptome analyses with several advantages, including the ability to detect and quantify transcripts, high sensitivity for low-abundance transcripts and single nucleotide resolution. Several transcriptomic studies performed on laboratory, industrial and genetically engineered *S. cerevisiae* strains revealed integrated approaches for cell survival under industrial and environmental stress conditions, including inhibitory compound and increased temperature exposure. These studies indicate that stress tolerance in *S. cerevisiae* is controlled by multiple loci that are widely distributed throughout the yeast genome.

An increase in temperature elicits a multitude of differentially expressed genes (DEGs). Genes significantly upregulated include those involved in protein catabolic processes, ubiquitin-dependent protein catabolic processes, proteolysis, protein folding and stress-related genes. Up-regulated genes involved in protein folding and refolding (*HSP10, HSP26, HSP60, HSP78, HSP82, HSP104, SSA1-4, SSE1* and *SSE2*) allow for the correct folding of denatured proteins (Boy-Marcotte et al. 1999; Yamamoto et al. 2008; Ismail et al. 2013). The increased expression of genes involved in ubiquitin-dependent protein catabolic processes (*HUL5, UBC4, UBC6, UBP3, UBP6* and *UBP9*) assist in the degradation of denatured proteins (Boy-Marcotte et al. 1999; Yamamoto et al. 2008; Ismail et al.

2013). Yang et al. 2013 identified two causative genes (*PRP42* and *SMD2*) involved in thermotolerance that are both involved in pre-mRNA splicing, suggesting an important role for RNA processing in conferring thermotolerance. Prp42 is an essential protein for U1 small nuclear ribonucleoprotein (snRNP) biogenesis, whereas SmD2 is part of the spliceosomal U1, U2, U4 and U5 snRNPs. These snRNPs function in pre-mRNA splicing by recognizing short conserved sequences from 59 to 39 nucleotides at the exon-intron junctions and assemble into active spliceosomes. In addition, *MKT1* has been identified as a causative gene in several QTL mapping studies for various phenotypes. Mkt1 appears to control gene expression at a post-transcriptional step and its deficiency produce effects on a range of diverse phenotypes (Yang et al. 2013). Expression of general stress-regulated genes (*GRE2*, *GRE3*, *INO1*, *STI1*, *SPG4* and *YGK3*) is also upregulated (Ismail et al. 2013). These genes are normally upregulated in response to osmotic-, ionic-, oxidative-, heat- and heavy metal exposure. Down-regulated genes include genes involved in metabolic processes such as amino acid-, amine-, carboxylic acid- and organic acid metabolic processes (Zhao and Bai 2009; Ismail et al. 2013). In addition, the cytoplasmic and ribosomal protein genes are repressed to conserve energy, allowing cells to maintain protein translation and folding at the expense of increased protein synthesis (Zhao and Bai 2009; Ismail et al. 2013). Oxygen requirements increase at high temperatures as the cells need to increase ATP generation. This leads to a decrease in ergosterol gene expression, which is important for cell viability and resistance to ethanol. Furthermore, thermotolerant yeast may also elicit a HSP-independent process that relies on trehalose accumulation (Lu et al. 2012)

Exposure to multiple inhibitory compounds elicits several differentially expressed genes. These include genes involved in general cell metabolism (carbon, fatty acid, alanine, aspartate and glutamate metabolism), genes associated with the cell wall, cell membranes and the mitochondria, genes involved in transport and stress responses (DNA and oxidative stress) and genes involved in signalling pathways (MAPK and PKA signalling pathways) (Zhao et al. 2015; Thompson et al. 2016). Inhibitory compounds are detoxified by the increased production of several dehydrogenases (ALD2/3, ADH1/6 and FDH1) (Liu 2011; Thompson et al. 2016). This detoxification requires reductive processes, leading to an imbalance in the cellular redox potential. To maintain the oxidative/reductive balance, the metabolic flux through several metabolic processes are

adjusted. Upregulation of the pentose phosphate pathway (PPP) provides reducing equivalents to maintain the redox potential of the cell (Herrero et al. 2008).

Alteration of the amino acid biosynthetic pathway allows for rapid ATP regeneration via the tricarboxylic acid (TCA) cycle (Zhao et al. 2015; Thompson et al. 2016). The upregulation of the TCA cycle restores the redox balance while maintaining energy production (Liu 2011; Thompson et al. 2016). Upregulation of fatty acid degradation via the peroxisomes increases the flux through the beta-oxidation pathways, providing energy and intermediates for cell growth (Zhao et al. 2015). The MAP kinase signalling pathway assists cell survival during oxidation stress by reprogramming metabolic pathways, including cell cycle, cell wall modification, energy metabolism, anti-oxidant biosynthesis, glutamate metabolism and amino acids biosynthesis (Zhao et al. 2015). The overexpression of multidrug transporter genes (*PDR10, PXA2,* and *SGE1*) enhance tolerance to inhibitors as it facilitates the removal of inhibitors from inside the cell (Liu 2011; Thompson et al. 2016). Maintaining cell wall and membrane integrity is essential when cells are exposed to cellulosic derived inhibitors as these compounds disrupt cellular membranes. Genes *CHS5, FLC1, HES1, PIR1* and *PUN1* are important for cell wall biosynthesis and function and their expression is upregulated when cells are exposed to inhibitory compounds (Zhao and Bai 2009; Thompson et al. 2016). Inhibitory compounds also cause DNA damage hence the observed change in expression of genes implicated in DNA repair and maintenance including (*DDI1, ENO1, IWR1, MAG1, TFS1* and *TPS2*) (Zhu and Xiao 2004; Thompson et al. 2016). Mitochondrial DNA is particularly sensitive to oxidative damage and expression of genes involved in the repair and protection of mitochondrial DNA (*DLD3*, *ETR1*, *MKS1, MSH1*, *OLE1,* and *PIR1*) is increased upon inhibitor exposure include (Thompson et al. 2016).

In this study, we analysed the complete transcriptome of two natural *S. cerevisiae* strains YI13 and YI30, capable of fermenting at high temperatures and in the presence of a synthetic inhibitor cocktail, respectively. The transcriptomic data of the two yeast strains was used to elucidate the molecular mechanisms that contribute to the maintained fermentative capacity of the different strains exposed to an increase in temperature and a synthetic inhibitor cocktail. The two strains were identified in a previous study where we compared the physiological differences and fermentation capacity of several natural strains (Jansen et al. 2018). Fermentation experiments were

performed at 30 °C and 37 °C and in the absence or presence of a 15 % synthetic inhibitor cocktail, respectively, whereafter RNA profiling was used to explore differential gene expression under relaxed and induced conditions.

MATERIALS AND METHODS

Strains

Natural *S. cerevisiae* strains YI13 and YI30 identified as thermo- and inhibitor tolerant, respectively (Jansen et al. 2018), were used as they maintained their fermentative capacity. Strains were propagated in YPD media and all experiments were performed in biological quadruplicates.

Glucose Fermentations

Fermentation experiments were performed as described in Jansen et al. (2018). Briefly, overnight cultures grown in MNS medium (Delfini et al. 1992) with 20 % glucose (w/v) were inoculated (7.5 x 10^4 cells per mL) into serum bottles containing 100 mL MNS medium. Yeast cells were harvested two hours after inoculation, whereafter cell pellets were flash-frozen in liquid nitrogen and stored at -80 °C. Two sets of fermentation experiments were performed: temperature tolerance fermentations were performed at 30 °C (control) and 37 °C and to evaluate inhibitor tolerance, fermentations were performed in the absence (control) and presence of a 15 % synthetic inhibitor cocktail.

RNA Isolation and Sequencing

Total RNA was isolated using the RiboPure™-Yeast Kit (Ambion, Life Technologies, Thermo-Fisher Scientific, USA) as per manufacturer's instructions. RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyser 2100 system (Agilent Technologies, CA, USA). Two methods were used to determine RNA quality and concentration, namely a Qubit RNA Assay Kit with a Qubit 2.0 Fluorometer (Life Technologies, CA, USA) and using the ND-1000 UV-visible light spectrophotometer (NanoDrop Technologies). High-throughput RNA sequencing was performed using the Illumina NextSeq platform (Nucleomics Core Institute, VIB, Belgium). The TruSeq RNA sample preparation kit (Illumina, San Diego, CA, USA) was used to construct a cDNA library. The fragmented first strand cDNA libraries of each sample were sequenced (2 × 75 cycles) using the NextSeq High Output kit.

Pre-processing and Quality Assurance of RNA Sequencing Data

The raw fastq-files (RNAseq) were quality filtered by trimming low quality read ends, removal of low quality reads and reads shorter than 35 bp with FastX 0.0.14 (HannonLab, Fastx-toolkit, http://hannonlab.cshl.edu/fastx_toolkit), ShortRead 1.24.0 and Cutadapt 1.7.1 (Gentleman et al. 2004; Martin 2011). The 5' and 3' ends of RNAseq reads were trimmed according to the Phred quality score (threshold < Q20) using ShortRead 1.24.0 R (Morgan et al. 2009). Adapter trimming (10 bp overlap and 90 % match) were performed with Cutadapt 1.7.1 to remove reads shorter than 35 bp (Martin 2011). Pairing consistency was assessed and broken pairs removed. The *S. cerevisiae* strain S288c was used as a reference genome for RNAseq read alignment. Reads that aligned to the phix_illumina control were removed via alignment with Bowtie 2.2.4 (Langmead and Salzberg 2012).

Transcriptome Analysis

RNAseq reads were aligned to the *S. cerevisiae* strain S288c reference genome with Tophat v2.0.13 (Trapnell et al. 2009). Samtools 1.1 were used to remove non-primary and low quality (<20) mappings and to sort alignments according to chromosomes (Li et al. 2009).

Gene Expression

Gene expression levels were assessed using the criteria described below. Counting per gene was determined using featureCounts 1.4.6 (Liao et al. 2014). Merging with gene annotation was included. Transcripts were filtered and absent transcripts (transcripts for which all samples have less than 1 counts-per-million) were removed (Robinson and Smyth 2007). Within-sample normalization (using full quantile normalization on bins of GC-content) (Risso et al. 2011) as well as between-sample normalization (library size and RNA composition) (Robinson and Oshlack 2010) were determined with the EDASeq R package (Risso et al. 2011). For each sample, the FPKM (number of fragments per kilobase of gene sequence and per million fragments) value were determined.

Determination of Differentially Expressed Genes (DEGs)

Four criteria were used to identify DEGs: Statistical modelling [fitting a negative binomial generalized linear model (GLM)] (Robinson and Smyth 2007) and hypothesis testing (using the model estimates with a GLM likelihood ratio test) was performed using the EdgeR 3.8.6 package (Chen et al. 2015). Correction for multiple testing was performed to control the false discovery rate (FDR) using the EdgeR package with

Benjamini-Hochberg correction (Benjamini and Hochberg 1995). Finally, DEGs were selected by combining the false discovery rate (FDR < 0.01) and statistical power ($p < 0.05$) (MAQC Consortium 2006).

Gene Ontology Data Analysis

The R 3.4.1 statistical programming language was used to filter DEGs based on a log-fold change (logFC) value of +/-1 and a FDR value threshold of 1 % (0.01). Gene ontology (GO) analysis was performed using the PANTHER database (Mi et al. 2016), AmiGO Term Enrichment Service (MAQC Consortium 2006; Carbon et al. 2009) and REVIGO (doi:10.1371/journal.pone.0021800). Data were condensed using GO slim mapping.

RESULTS

The transcriptome of two natural *S. cerevisiae* strains exposed to two different environments were determined using the RNA-seq platform. *Saccharomyces cerevisiae* strains YI13 and YI30 were previously identified as temperature and inhibitor tolerant strains, respectively (Jansen et al. 2018). In order to elucidate the molecular mechanisms responsible for these tolerances, the transcriptome of these strains was determined two hours exposure to an increased temperature (37 °C) and synthetic inhibitor cocktail (15 %), respectively. The experimental design was specific to determine the yeast strains' initial response to stressors, as the fermentation process in itself is associated with various stressors that change and/or accumulate as the fermentation reaction progresses.

RNA Isolation and Sequence Analysis

The natural *S. cerevisiae* strains, YI13 and YI30, were cultivated under relaxed or induced conditions. Two hours after exposure to the specific environmental condition (increased temperature or inhibitor cocktail), the fermentation reaction was terminated through flash freezing in liquid nitrogen and RNA was isolated to determine the transcriptome at the early induction phase. High-quality RNA with an $A_{260/280nm}$ ratio of above 2.1 was obtained with only one sample with an $A_{260/230nm}$ ratio below 1.8 ($A_{260/230nm}$ ratio 1.13). On average, the high to low molecular weight rRNA ratio was equal to two and the bioanalyser assessment yielded a RIN value >7. Multivariate statistical analysis of RNAseq data using principal component analysis (PCA) was used to determine the comparative transcriptome datasets of the different strains. This is represented on a two-dimensional graph (Figure 1) using the two first principal components that display

the biological variation between the samples. The samples represent two separate sets of fermentations (control and experimental condition) for two different strains (temperature tolerant YI13 and inhibitor tolerant YI30). Figure 1 indicates a significant difference between the two *S. cerevisiae* strains, as well as between the relaxed and the induced experimental conditions. The variance between the biological repeats are also indicated.

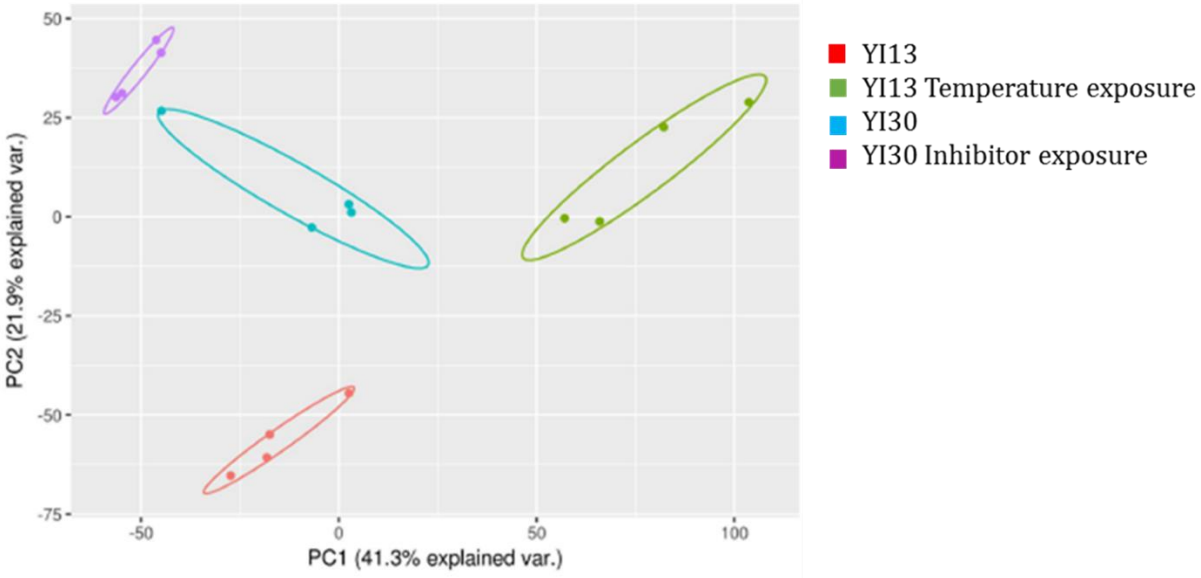

Figure 1: Principal component analysis (PCA) to determine the biological variation between samples and experimental conditions. The data ellipses indicate a 95% confidence interval between the individual samples for the strains tested, as well as for each experimental condition tested. Data was normalised to remove technical artefacts.

Determination of Differentially Expressed Genes (DEGs)

The amount of differentially expressed genes with statistical significance ($\log_2$-ratios versus minus the $\log_{10}$ p-value) were calculated using edgeR and are represented as volcano plots (Figure 2). The magnitude of differential expression ($\log_2$ fold-change) is compared to the measure of statistical significance ($-\log_{10}$ q-value). Genes passing a FDR <0.01 and a log fold change >1 threshold were regarded as significantly differentially expressed. As indicated in Figure 2, there is an even distribution of differentially expressed genes. The temperature tolerant *S. cerevisiae* strain YI13 displayed a total number of 724 significantly DEGs (FDR <0.01) at log fold ratio greater than 1, of which 399 genes were upregulated and 325 genes were downregulated. In comparison, the inhibitor tolerant *S. cerevisiae* strain YI30 displayed a total number of 656 significantly

DEGs (FDR <0.01) at log fold ratio greater than 1, with 382 genes upregulated and 274 genes downregulated.

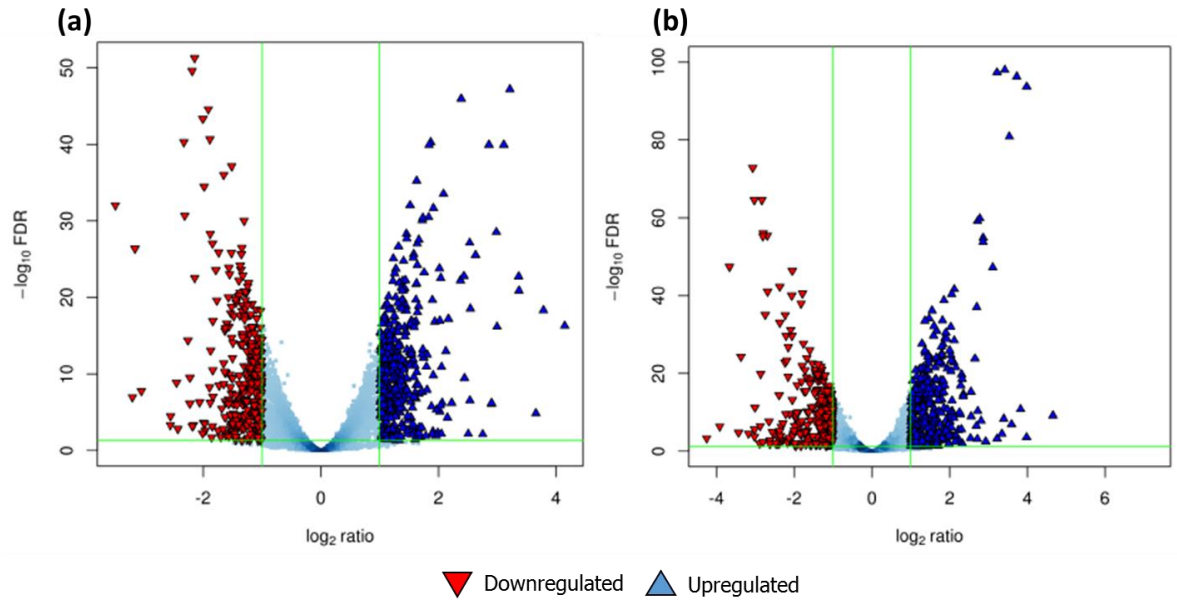

Figure 2: Volcano plots using the FDR-values vs the fold change was constructed for each of the two experiments. Data for the temperature tolerant *S. cerevisiae* strain YI13 and the inhibitor tolerant *S. cerevisiae* strain YI30 are represented in graphs (a) and (b) respectively, with red and blue dots representing down- and up-regulated genes, respectively. The magnitude of differential expression indicated by the ratio ($\log_2$ fold-change) compared to the measure of statistical significance indicated by the false discovery rates (FDR) ($-\log_{10}$ q-value) is used to determine the statistical significance of gene transcription. Statistically significant differentially expressed genes are observed above the green horizontal line indicating the $\log_2$ fold-change threshold and on either side of the vertical green lines indicating the significance threshold (0.01 FDR), which corresponds to a p-value of 0.05.

Gene Ontology Data Analysis

Gene Ontology (GO) analysis was performed to group the data obtained from the RNAseq experiments into functional gene clusters. The relevant GO processes are divided into three categories: biological processes (BP), cellular components (CC) and molecular functions (MF). The percentage of genes per category is presented as a pie chart, with additional pie charts to indicate the number of transcripts per GO term. The GO analysis for the temperature tolerant *S. cerevisiae* strain YI13 and the inhibitor tolerant *S. cerevisiae* strain YI30 is represented in Figures 3 (a) and (b), respectively.

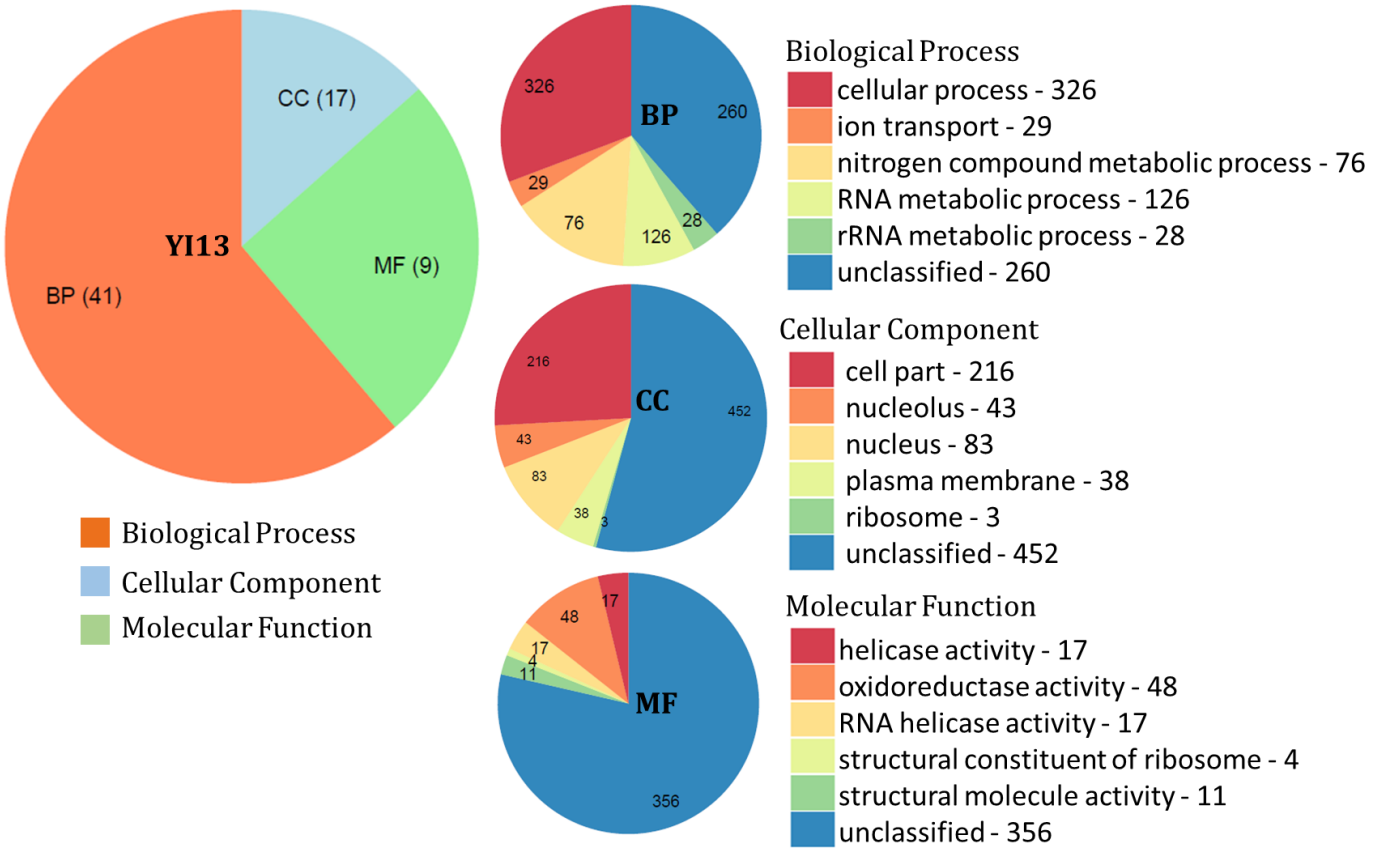

Figure 3 (a): GO data analysis of differentially expressed genes (DEGs) identified from comparative transcriptomic data analysis for the temperature tolerant *S. cerevisiae* strain YI13. Functional classification of genes is based on biological processes, molecular functions and cellular components. The number in brackets indicate the percentage of transcripts (number of transcripts involved in process out of total number of transcripts identified) identified per classification. For each classification, the number of transcripts identified for each specific function is indicated.

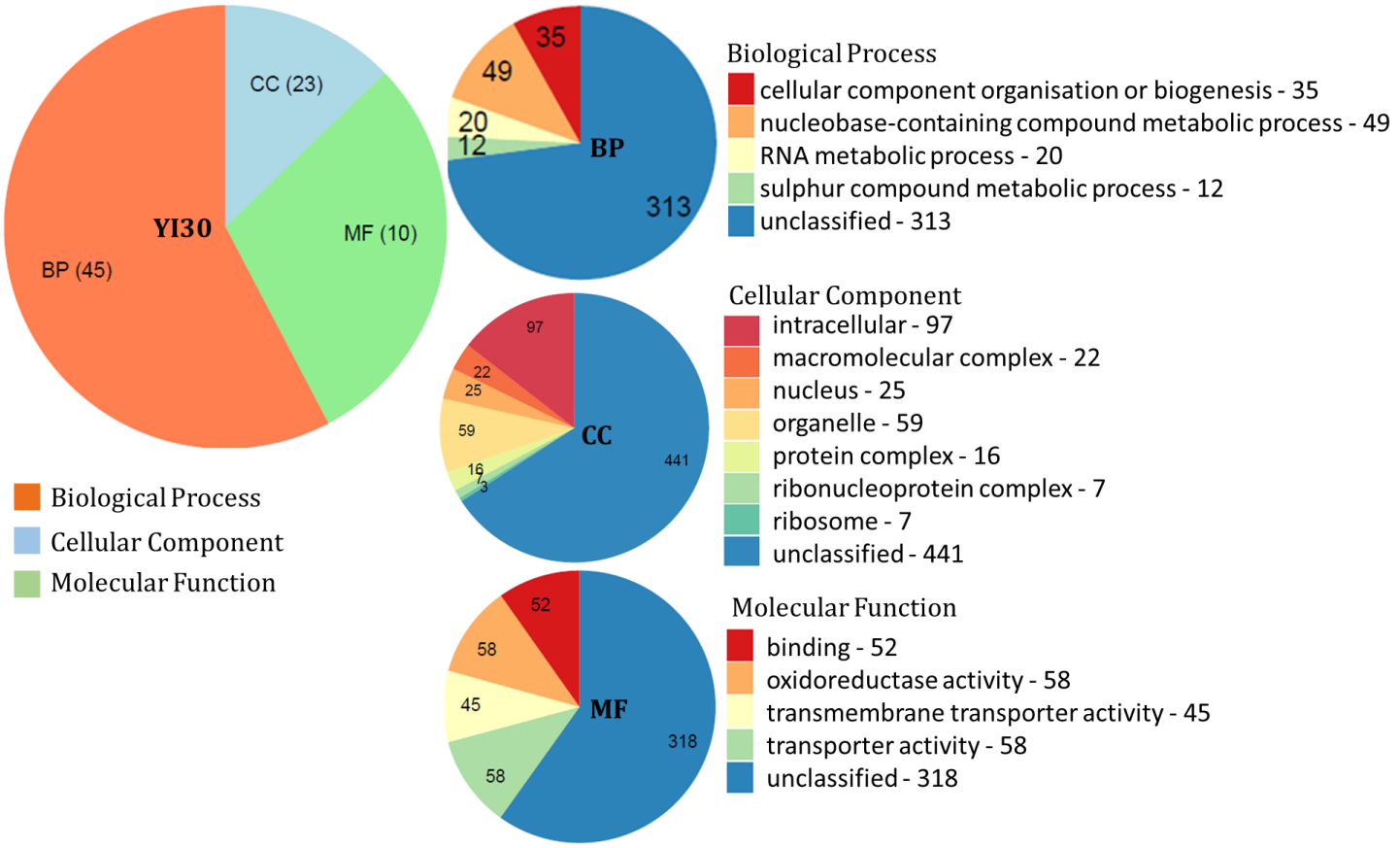

Figure 3 (b): GO data analysis of differentially expressed genes (DEGs) identified from comparative transcriptomic data analysis for the inhibitor tolerant *S. cerevisiae* strain YI30. Functional classification of genes is based on biological processes, molecular functions and cellular components. The number in brackets indicate the percentage of transcripts (number of transcripts involved in process out of total number of transcripts identified) identified per classification. For each classification, the number of transcripts identified for each specific function is indicated.

Biological processes (41 %) were most affected in the temperature tolerant strain YI13, followed by cellular components (17 %) and molecular functions (9 %) (Figure 3 a). For the biological processes, the majority of differentially expressed transcripts are associated with cellular processes (326), followed by RNA metabolic processes (126), nitrogen compound metabolic processes (76), ion transport (29) and rRNA metabolic processes (28), with 260 unclassified transcripts. For the cellular components, the majority of differentially expressed transcripts are associated with cell parts (216), followed by nucleus (83), nucleolus (43), plasma membrane (38) and ribosome (3), with 452 unclassified transcripts. The majority of differentially expressed transcripts for the molecular function are associated with oxidoreductase activity (48), with helicase and RNA helicase activity having the same number of transcripts (17) followed by structural molecule activity (11) and structural constituent of ribosome (4), with 356 unclassified transcripts. Figure 3 (b) represents the GO analysis for the inhibitor tolerant *S. cerevisiae* strain YI30. As with the thermotolerant strain, biological processes (41 %) were most affected followed by cellular component (23 %) and molecular function (10 %). The most significant number of differentially expressed transcripts for the biological processes are associated with nucleobase-containing compound metabolic processes (49), followed by cellular component organisation or biogenesis (35), RNA metabolic processes (20) and sulphur compound metabolic processes (12), with 312 unclassified transcripts. The most significant number of differentially expressed transcripts for the cellular component are associated with intracellular (97), followed by organelle (59), nucleus (25), macromolecular complex (22), protein complex (16), ribonucleoprotein complex (7) and ribosome (3), with 441 unclassified transcripts. As for the molecular function, the most significant number of differentially expressed transcripts are associated with both oxidoreductase and transport activity (both with 58 transcripts), followed by binding (52) and transmembrane transporter activity (45), with 318 unclassified transcripts.

To establish the relevance of the processes that displayed DEG, the most significant GO terms were determined using Revigo (http://revigo.irb.hr/revigo.jsp) (Supek et al. 2011). The most relevant GO processes for the temperature tolerant *S. cerevisiae* strain YI13 and the inhibitor tolerant *S. cerevisiae* strain YI30 are represented in Figures 4 (a) and (b), respectively. The three categories (BP, CC and MF) were sub-divided into

specific processes, structures and functions representing the most relevant processes affected by the specific experimental condition tested. The biological processes enriched for in the temperature tolerant strain *S. cerevisiae* YI13 when exposed to an increase in temperature, were associated with RNA metabolic processes and ribosome biogenesis with the nucleolus and the pre-ribosome the most significantly enriched cellular component, and the structural constituent of the ribosome and the structural molecule activity the most relevant molecular functions.

The processes and functions of each of the three GO processes are related as rRNA processing and ribosome biogenesis take place in the nucleolus. When assessing the inhibitor tolerant *S. cerevisiae* strain YI30 after exposure to a synthetic inhibitor cocktail, the most relevant biological processes are associated with macromolecule metabolic processes, followed by RNA processing. The macromolecular group is the most relevant cellular component followed by the catalytic complex with oxidoreductase and structural molecule activity the most relevant molecular functions. There is a link between the different GO categories as macromolecule metabolic processes occur in the cytosol with oxidoreductase activity occurring in both the mitochondrion and the cytosol. The *S. cerevisiae* laboratory strain S288c that was used as reference strain, is not fully annotated, with 11 % uncharacterised and 10 % dubious genes. In addition, although extremely rare, natural *S. cerevisiae* strains may contain non-S288c ORFs that were possibly obtained from the environment (Novo et al. 2009; Fitzpatrick 2012; Coelho et al. 2013). Therefore, several differentially expressed transcripts remain uncharacterised.

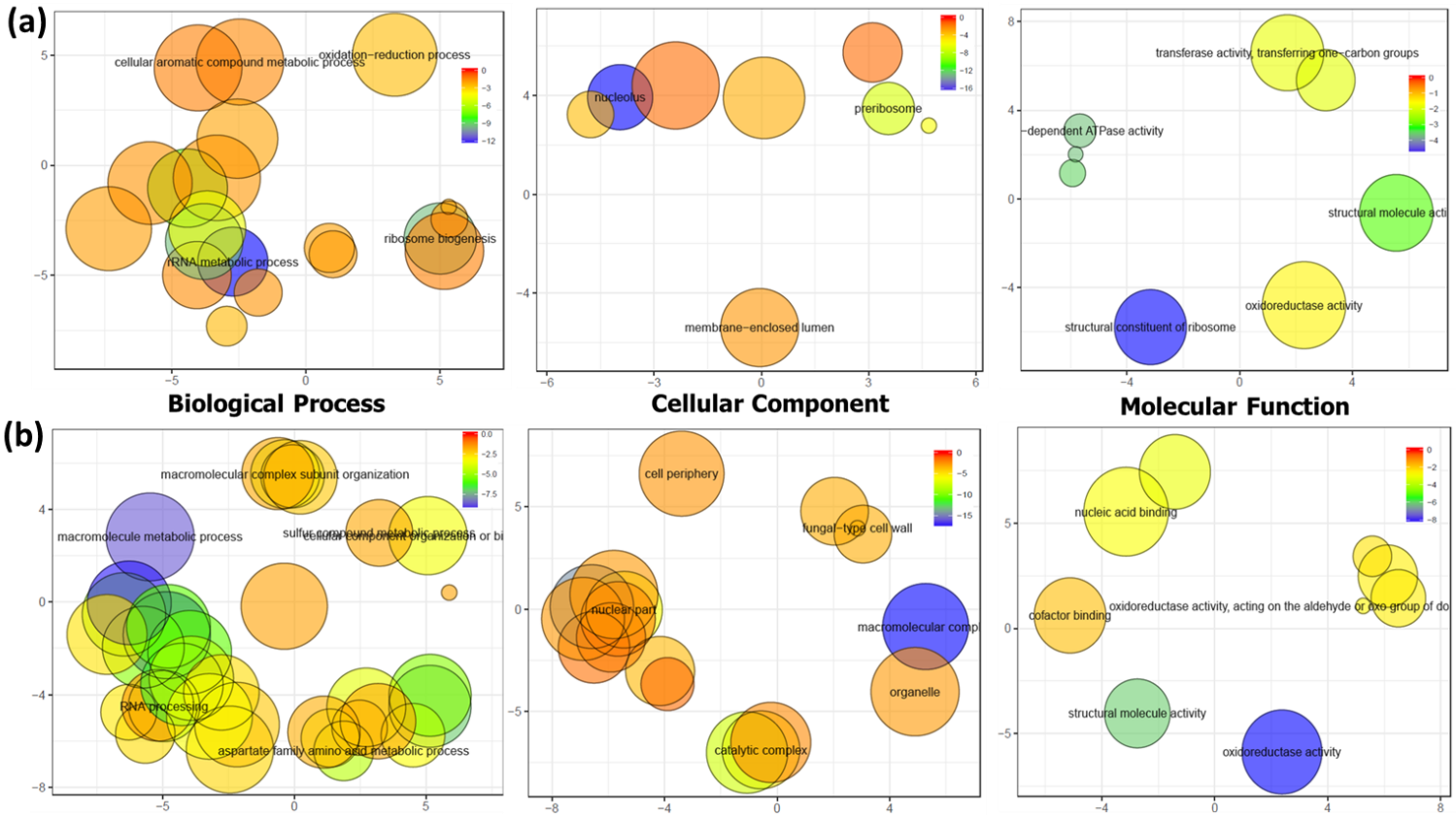

Figure 4: PCA plots using Revigo was used to determine the most relevant processes influenced by DEGS for (a) the temperature tolerant *S. cerevisiae* strain YI13 and (b) the inhibitor tolerant *S. cerevisiae* strain YI30. The GO terms are visualised in a semantic space (y- and x-axis) where similar terms are positioned closer together. The colour (red = high, green = moderate, blue = low) of the bubble reflects the p-value obtained in the GOrilla analysis, whereas the size (big = high, small = low) reflects the generality of the GO term in the UniProt-GOA database.

The top twenty DEGs (based on log fold change) for the temperature and inhibitor experiments are indicated in Tables 1 and 2, respectively, and included several uncharacterised genes (seven and five, for the thermo- and inhibitor tolerant strains, respectively). Of the top 10 upregulated genes for the thermotolerant strain YI13 (Table 1), the majority of the genes are involved in amino acid biosynthesis (*MUP1, VBA2* and *JLP1*) and DNA remodelling processes (*SAE3* and *TTI2*). In addition, the cell wall mannoprotein *PAU24* plays a role in maintaining cell wall and membrane structure and integrity during stress conditions, which is a direct response to an increase in temperature to maintain cell wall integrity. *PCL10* encodes for a Pho85 cyclin that is involved in glycogen biosynthesis, suggesting a role in temperature stress tolerance (Huang et al. 1998). Among the top 10 downregulated genes, one is implicated in spore formation (*LDS1*) whereas two genes play a role in respiration (*ACO1* and *PDH1*). The most down-regulated gene (*BTN2*) is a direct target of HSF1 and has been previously implicated in ethanol tolerance (Espinazo-Romeu et al. 2008). The highly downregulated gene *YNL284C-B* is implicated in DNA remodelling, whereas *YAR066W* encodes a possible cell wall protein.

The majority of the top ten upregulated genes for the inhibitor tolerant strain (Table 2) encode for oxidoreductases (*YKL071W*, *ADH7* and *YPR127W*) and transport proteins (*PDR12* and *FLR1*), suggesting that inhibitor tolerance is due to inhibitor detoxification and removal of the inhibitory compounds. Six of the ten most downregulated genes encode for tRNAs (*TGA1*, *SUF4*, *tF(GAA)P2*, *SUF6*, *tG(GCC)E* and *tA(UGC)E*), indicating a downregulation of translation and therefore protein synthesis. In addition, two of the top downregulated genes encode for proteins involved in mitochondrial processes (*AAC3* and *CTP1*). The highly downregulated genes *MET5* and *FET4* encode for proteins involved in amino acid biosynthesis and iron transport, respectively, confirming the importance of downregulation of protein synthesis in inhibitor tolerance.

Table 1: Highly DEGs of *S. cerevisiae* strain YI13 in response to temperature

Gene	Description	Log-Fold Change
YER090C-A	Uncharacterised protein	4.15
SAE3	Meiosis-specific protein involved in meiotic recombination	3.78
PAU24	Cell wall mannoprotein	3.66
TTI2	Subunit of ASTRA complex, involved in chromatin remodelling	3.37
MUP1	High affinity methionine permease	3.36
VBA2	Permease of basic amino acids in vacuolar membrane	3.21
JLP1	Fe(II)-dependent sulfonate/alpha-ketoglutarate dioxygenase	3.11
YOR381W-A	Uncharacterised protein	3.00
PCL10	Pho85p cyclin	2.98
YBR300C	Uncharacterised protein	2.90
BTN2	v-SNARE binding protein	-3.49
YAR066W	Putative GPI protein	-3.20
PDH1	Putative 2-methylcitrate dehydratase	-3.16
YGR069W	Uncharacterised protein	-3.05
YGL063C-A	Uncharacterised protein	-2.56
LDS1	Protein involved in spore wall assembly	-2.55
YOL085C	Uncharacterised protein	-2.45
YDR524W-C	Uncharacterised protein	-2.43
YNL284C-B	Retrotransposon TYA Gag and TYB Pol genes	-2.33
ACO1	Aconitase	-2.31

Table 2: Highly DEGs of *S. cerevisiae* YI30 in response to inhibitor cocktail

Gene	Description	Log-Fold Change
YKL071W	Aldehyde reductase	7.22
ADH7	NADPH-dependent medium chain alcohol dehydrogenase	6.41
YML122C	Uncharacterised protein	4.66
YKL070W	Uncharacterised protein	4.35
PDR12	Plasma membrane ATP-binding cassette (ABC) transporter	3.98
YJL028W	Uncharacterised protein	3.98
YER152W-A	Uncharacterised protein	3.83
FLR1	Plasma membrane transporter	3.73
YLR111W	Uncharacterised protein	3.60
YPR127W	Putative pyridoxine 4-dehydrogenase	3.54
TGA1	Alanine tRNA	-4.25
SUF4	Glycine tRNA	-3.91
AAC3	Mitochondrial inner membrane ADP/ATP translocator	-3.66
tF(GAA)P2	Phenylalanine tRNA	-3.43
CTP1	Mitochondrial inner membrane citrate transporter	-3.37
SUF6	Glycine tRNA	-3.17
MET5	Sulphite reductase beta subunit	-3.07
FET4	Low-affinity Fe(II) transporter of the plasma membrane	-3.02
tG(GCC)E	Glycine tRNA	-3.02
tA(UGC)E	Alanine tRNA	-3.02

DISCUSSION

Metabolically active organisms require the co-ordinated regulation of several pathways in order to maintain viability. These include energy and metabolism, cell cycle regulation, cellular transport, cell rescue, cell defence and protein synthesis. The two natural strains used in this study were selected for their fermentative capacity when exposed to high temperatures (*S. cerevisiae* strain YI13) and lignocellulosic inhibitors (*S. cerevisiae* strain YI30). Fermentation is characterized by high rates of fermentative metabolism associated with a reduction in growth and biomass formation (Rossouw et al. 2010). The core metabolic activities during fermentation include hexose metabolism, glycolysis, trehalose metabolism and redox balance. The genes involved in these key metabolic pathways are therefore highly induced and we expected to observe transcriptional reprogramming of several of the genes involved in these core metabolic activities when exposed to higher temperatures and lignocellulosic inhibitors.

General Responses Observed in Both Natural Strains

TPS3, a regulatory subunit of trehalose-6-phosphate synthase/phosphatase, was downregulated in the thermotolerant strain, YI13 whereas several genes (*NTH1*, *TPS2* and *TSL1*) were upregulated in the inhibitor tolerant strain YI30. Metabolic restructuring in carbon metabolism during fermentation is responsible for changes in the expression of genes involved in trehalose metabolism as trehalose is an important stress metabolite and allosteric regulator of several important glycolytic enzymes (Rossouw et al. 2009). Trehalose-6-phosphate determines the flux through glycolysis and provides energy and intermediates for fermentation, glycerol metabolism and the oxidative pentose phosphate pathway (Rossouw et al. 2009). In addition, trehalose accumulation has been identified as an important contributing factor for increased thermo-, ethanol and osmotic tolerance.

Various genes involved in protection against oxidative stress are typically upregulated during fermentation to maintain the redox potential (Rossignol et al. 2003; Rossouw et al. 2009). Several dehydrogenases (*ADH5*, *GND1*, *GPD1*, *IDH1*, *IDH2*, *IDP1* and *NDE1*) and reductases (*ARI1*, *GRX2*, *QCR10* and *YHB1*) are downregulated in the thermotolerant strain YI13, whereas dehydrogenases (*ADH6*, *ADH7*, *FDH1*, *GCY1*, *GDH2*, *GND2*, *TDH1*, *SFA1* and *YNL134C*) and reductases (*ARI1*, *FRM2*, *GRE2*, *GRE3*, *GRX1*, *GRX2*, *OYE3*, *QCR8*, *YDL124W*, *YHB1*, *YJR096W*, *YML131W* and *ZTA1*) were upregulated in the inhibitor tolerant strain YI30. *AAD* genes, which encode putative aryl-alcohol dehydrogenases, are normally upregulated in response to oxidative stress (Sundström et al. 2010). Several *AAD* genes (*AAD3*, *AAD4* and *AAD10*) were upregulated in the inhibitor tolerant strain, whereas only *AAD3* was upregulated in the temperature tolerant strain, suggesting that inhibitors exacerbate the oxidative stress experienced by the yeast. In addition, *ALD 3* was induced in the inhibitor tolerant strain, whilst the downregulation of *ALD6* (involved in acetate formation) was evident.

Components involved in protein synthesis, including ribosomal proteins, translation factors, rRNA transcription and processing, tRNA synthesis and aminoacyl-tRNA-synthase, are normally downregulated during fermentation (Rossignol et al. 2003). This is observed in the inhibitor tolerant strain, but we observed an induction in the transcription of these genes in the temperature tolerant strain with genes involved in rRNA transcription, processing and ribosome biogenesis significantly upregulated. In

addition, an upregulation of the classical stress-responsive heat shock genes (*HSP42, HSP78, HSP104* and *SSE2*), which encode proteins involved in protein folding, is generally observed during fermentation and was observed in the inhibitor tolerant strain (Rossignol et al. 2003). However, these transcripts were downregulated in the temperature tolerant strain. These observations suggest that the normal stress response associated with fermentation and observed in the inhibitor tolerant strain do not compare with the response observed in the temperature tolerant strain. The transcriptome of the temperature tolerant strain did not show the typical response to fermentation, suggesting a possible superior tolerance to fermentation stress in the presence of an increased fermentation temperature.

Temperature Tolerant Strain YI13

A global overview of the transcriptomic data in response to an increase in temperature suggests that cellular and RNA metabolic processes are amongst the most highly enriched biological processes [Figure 3 (a) and 4 (a)]. The most relevant processes include rRNA metabolic processes with the nucleolus and the structural ribosome constituent being the most affected cellular compartment molecular function, respectively. Oxidoreductase activity is the molecular mechanism most affected during fermentation at the higher temperature. Oxidative stress is normally associated with the fermentation process and an increase in temperature may exacerbate the oxidative stress response due to the increased formation of ROS species at the higher temperature (Morano et al. 2012).

Transcriptional factors that were upregulated in strain YI13 included those involved in nucleotide and amino acid biosynthesis (*BAS1, GCN1, LEU3, MET4, MET32, PPR1* and *PZF1*). In addition, two of the most highly upregulated genes, *JLP1* and *VBA2*, are also associated with amino acid metabolism. *JLP1* is involved in sulphur amino acid metabolism (Fletcher et al. 2017) and *VBA2* encodes a vacuolar amino acid transporter that mediates histidine, arginine, tyrosine and lysine uptake (Shimazu et al. 2005). This suggests an increased requirement for amino acid biosynthesis, which could indicate an alternative mechanism to maintain general protein stability. Yeast cells usually respond to protein damage by upregulating chaperone and protein-folding activity, whereas an increase in amino acid biosynthesis and ribosome biogenesis was observed in this study. The main function of ribosomes is to organize protein synthesis, suggesting that protein

synthesis may have been accelerated to maintain protein function. In addition, *PCL10* is implicated in glycogen biosynthesis and could play a role as a protectant against temperature stress (Huang et al. 1998).

The TTT (TEL2-TTI1-TTI2) complex associates with several molecular chaperones and plays a role in the general stress response (Hoffman et al. 2016). It is required to maintain steady-state levels of phosphatidylinositol 3-kinase-related kinase (PIKK) proteins, which serve as regulators of critical cell signalling pathways. Biosynthesis and regulation of PIKK proteins is essential for cells growth, proliferation, and the stress response. The highly upregulated *TTI2* gene in strain YI13 is one of three essential proteins to form the TTT complex and plays a role in protein biosynthesis (Hoffman et al. 2016). Furthermore, *TTI2* is also involved in chromatin remodelling and together with *SAE3*, which plays a role in meiotic recombination, may indicate a role in maintaining DNA integrity in response to DNA damage due to the increased temperature.

In addition to maintaining protein production, the general stress response observed in strain YI13 could be in response to oxygen limitation. Limited oxygen conditions inhibit the production of sterols required to maintain cell walls. An increase in temperature affects membrane integrity, resulting in the destabilisation of the cell wall. The upregulated *PAU24* encodes a cell wall mannoprotein and the increased expression may compensate for the decrease in sterol production to maintain cell wall integrity at elevated temperatures. The *PAU1-24* genes constitute the largest gene family in *S. cerevisiae* with 24 members (Luo and van Vuuren 2009). These genes are implicated during alcoholic fermentations with a specific role in maintaining cell wall integrity or sterol uptake during stress. The location of these genes in the subtelomeric regions of chromosomes suggests a role in the adaptation of yeast cells to stress as subtelomeric regions are hotspots for genetic recombination and strong evolutionary drivers (Luo and van Vuuren 2009; Dunn et al. 2012), and are implicated in copy number variation that could allow for the increase in transcript abundance.

Furthermore, *MUP1* encodes a high-affinity methionine permease that mediates methionine and cysteine uptake (García-Ríos et al. 2016). *MUP1* is also required for sulphur assimilation at low temperatures and has been shown to be upregulated when

cells are exposed to low temperatures (García-Ríos et al. 2016). *MUP1* is also upregulated during anaerobic conditions (Rintala et al. 2011). The decreased availability of oxygen may explain the upregulation of *MUP1* and oxidoreductases as indicated by the REVIGO gene ontology plots in Figure 3 (a) to maintain the cellular redox potential. Furthermore, an increase in temperature leads to an increase in ROS, further exacerbating the oxidative stress on the cell. Protein sulphydryl groups are especially sensitive to oxidation and form sulphonic derivatives through an irreversible process (Herrero et al. 2008). This may explain the upregulation of genes involved in the biosynthesis of specifically sulphur containing amino acids such as methionine and cysteine (*MET1, MET2, MET4, MET10, MET30, MET32 MHT1, MUP1, STR3* and *YCT1*). In addition, *ACO1*, a TCA cycle enzyme, is significantly downregulated when the fermentation temperature is increased, which may decrease the mitochondrial function and maintenance of the redox potential of the cell. Gene *BTN2*, the most significantly downregulated gene in strain YI13, is involved in several processes, including the control of the intracellular localization of different proteins, salt tolerance, ethanol tolerance, pH homeostasis and amino acid transport. Deletion of *BTN2* abolishes ethanol tolerance (Espinazo-Romeu et al. 2008).

The suggested *S. cerevisiae* strain YI13 transcriptional landscape in response to an increase in temperature is summarised in Figure 5. The increased temperature damages the cell wall, cell membranes, DNA and proteins. The temperature tolerant *S. cerevisiae* strain YI13 does not activate the usual stress response associated with protein damage, but presumably upregulates protein synthesis via amino acid biosynthesis and ribosome biogenesis to maintain protein integrity and thus cell viability. In addition, cell wall and membrane damage is contained by the upregulation of cell wall mannoproteins. The normal upregulation of sterol production to maintain cell membranes is prohibited, possibly due to the limited oxygen as an increase in temperature increases the oxygen requirements of the cell. Glycogen biosynthesis is upregulated as a general stress response to an increase in temperature. In addition, the oxidative stress is presumably regulated by upregulation of the TCA cycle to maintain the redox potential of the cell. Furthermore, the increase in temperature damages cell DNA, leading to an upregulation in DNA remodelling genes.

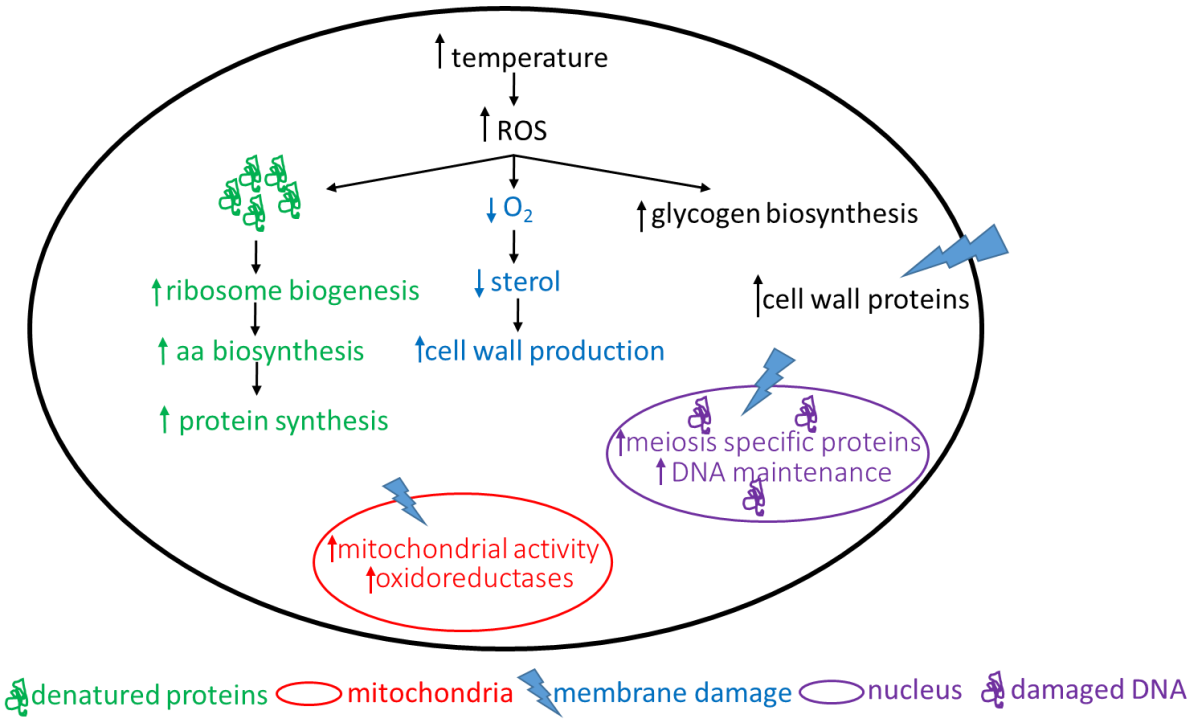

Figure 5: A schematic representation of the suggested mechanisms elicited by *S. cerevisiae* strain YI13 when the fermentation temperature is increased. An increase in temperature increases the production of reactive oxygen species (ROS), which damage the cell wall and cell membranes and denatures DNA and proteins. In response to the protein damage, protein synthesis is upregulated via amino acid biosynthesis and ribosome biogenesis in order to maintain protein integrity and thus cell viability. Cell wall and membrane protein synthesis is upregulated to maintain these structures as sterol production is prohibited due to the limited oxygen availability. Glycogen biosynthesis is upregulated as a protective measure against an increase in temperature. The oxidative stress is regulated by the mitochondria electron transport chain. DNA remodelling genes are upregulated to maintain DNA integrity.

Inhibitor Tolerant Strain YI30

Exposure to the synthetic inhibitory cocktail has a major effect on biological processes involving chemical and physical transformation. These include metabolic processes containing nucleobase compounds (nucleobases, nucleosides, nucleotides and nucleic acids) (Figure 3 b), such as cellular metabolic processes involving nucleobases, nucleosides, nucleotides and nucleic acids, reflected in the downregulation of several transcripts involved in these processes including *AAH1*, *PHM8*, *THI20*, *URH1* and *URA10*.

Purine and pyrimidine nucleotides are important energy carriers and play a major role in the synthesis of nucleotide cofactors such as NAD and SAM (Xu et al. 2013). Inhibitor exposure increases the energy and cofactor demand of the cell due to the increased metabolic flux via the TCA cycle and the PPP, and the oxidative stress experienced by the cell. In addition, ribose salvage is essential to cell survival during oxidative stress.

The second most affected biological process is the cellular component organisation or biogenesis, which encompasses processes that result in the biosynthesis of macromolecules, including assembly and arrangement or disassembly of a cellular component. It is suggested that cells adapt to inhibitor stress by downregulating the biosynthesis of macromolecules via the downregulation of protein synthesis. Cell growth (DEGs *CYK3, GPA1, HBT1, KEL2, MFA2, PFK1, PRY*3, *PRM4, RAS1, RIM18, SUT2, SWI5* and *TOS2*) is downregulated in order to divert cellular energy and components towards the detoxification processes.

Cellular components affected by exposure to cellulosic inhibitory compounds include the intracellular components and organelles. The detoxification process occurs within the cytosol of the cell. Inhibitory compounds are able to penetrate membranes, thus affecting organelle function (Klinke et al. 2004; Xie et al. 2016). Many organelles also experience an increase in activity, specifically the mitochondria due to increased flux via the TCA cycle (Liu 2011; Thompson et al. 2016).

Molecular functions that are most affected by inhibitor exposure, include molecular mechanisms involved in transport and oxidoreductase activity. The oxidoreductase activity probably relates to the detoxification of the inhibitory compounds. The detoxification process requires aldehyde and alcohol dehydrogenase, causing an upregulation of these transcripts (*AAD3, AAD4, AAD10, ADH6, ADH7, ALD3* and *SFA1*). The various dehydrogenases require the function of cofactors that are continually regenerated via the activity of the oxidoreductases, causing an upregulation of various oxidoreductases (*ARI1, CBY2, COX2, COX7, ERO1, FRM2, GRE2, GRE3, GPX1, GRX1, GRX2, OYE3, PRX1, QCR8, TRX2, YDL124W, YHB1, YJR096W, YML131W* and *ZTA1*). In addition, it is suggested that the inhibitory compounds that were able to penetrate the cell membrane are transported out of the cell, causing an upregulation of several multidrug

transporters (*ARR3, FLR1, PDR5, PDR12, PDR15, QDR1, VMR15, YDR061W* and *YMR034C*).

In contrast to *S. cerevisiae* strain YI13, the transcriptome of *S. cerevisiae* strain YI30 displayed a significant number of DEGs directly linked to exposure to the synthetic inhibitor cocktail. Several DEGs play a role in the detoxification of the inhibitory compounds. These include conversion of the compounds to less toxic compounds by alcohol dehydrogenases (*YCR105W, YCR107W, YDL168W, YDL243C* and *YJR155W*), including oxidoreductases (23 genes) that assist in generating the required reducing equivalents. In addition, 15 genes encoding transport proteins have been identified that assist in the removal of the inhibitory compounds. Moreover, genes implicated in the environmental stress response, including genes encoding heat shock proteins (*FES1, HSC82, HSP26, HSP42, HSP104, SSA4* and *SSE2*) involved in protein folding and degradation (19 genes), membrane proteins (16 genes) and genes involved in the trehalose biosynthesis pathway (*NTH1, NTH2, SPG1, SPG4, TPS2* and *TSL1*) are differentially expressed. Two genes (*DIA3* and *GPG1*) implicated in pseudohyphal growth are also upregulated in strain YI30 in response to inhibitor exposure. Pseudohyphal growth has been observed in yeast exposed to environmental stress, including nitrogen starvation (Zaragoza and Gancedo 2000; Gancedo 2001).

The metabolic flux through several pathways is reprogrammed to maintain cell viability in the presence of inhibitory compounds. Glycerol production is decreased (*ADR1, DGA1*, *GCY1, GPP1* and *YIG1*) in the presence of inhibitors during anaerobic conditions. Glycerol biosynthesis acts as a redox sink, providing additional reoxidation of cytosolic NADH. At the same time, NADH is the major cofactor required for reduction of inhibitors. As a result, glycerol production and inhibitor reduction compete for a shared pool of NADH (also observed for the reaction of acetaldehyde to ethanol and inhibitor reduction). Glycerol production is therefore reduced to meet the requirement of NADH for the conversion of furan aldehydes to less toxic alcohols. Furthermore, the PPP is an important carbohydrate metabolism pathway, oxidizing glucose to generate NADPH for reductive biosynthesis reactions within cells and ribose-5-phosphate for the synthesis of the nucleotides and nucleic acid. Upregulation of this pathway (*GND2*, *RKI1* and *TKL2*) is therefore required to maintain the redox potential of the cell.

Figure 6 depicts a schematic diagram of the suggested transcriptional landscape of *S. cerevisiae* strain YI30 when exposed to a synthetic inhibitory cocktail. This strain is able to maintain fermentation capacity in the presence of a synthetic inhibitor cocktail by utilising a combination of three mechanisms.

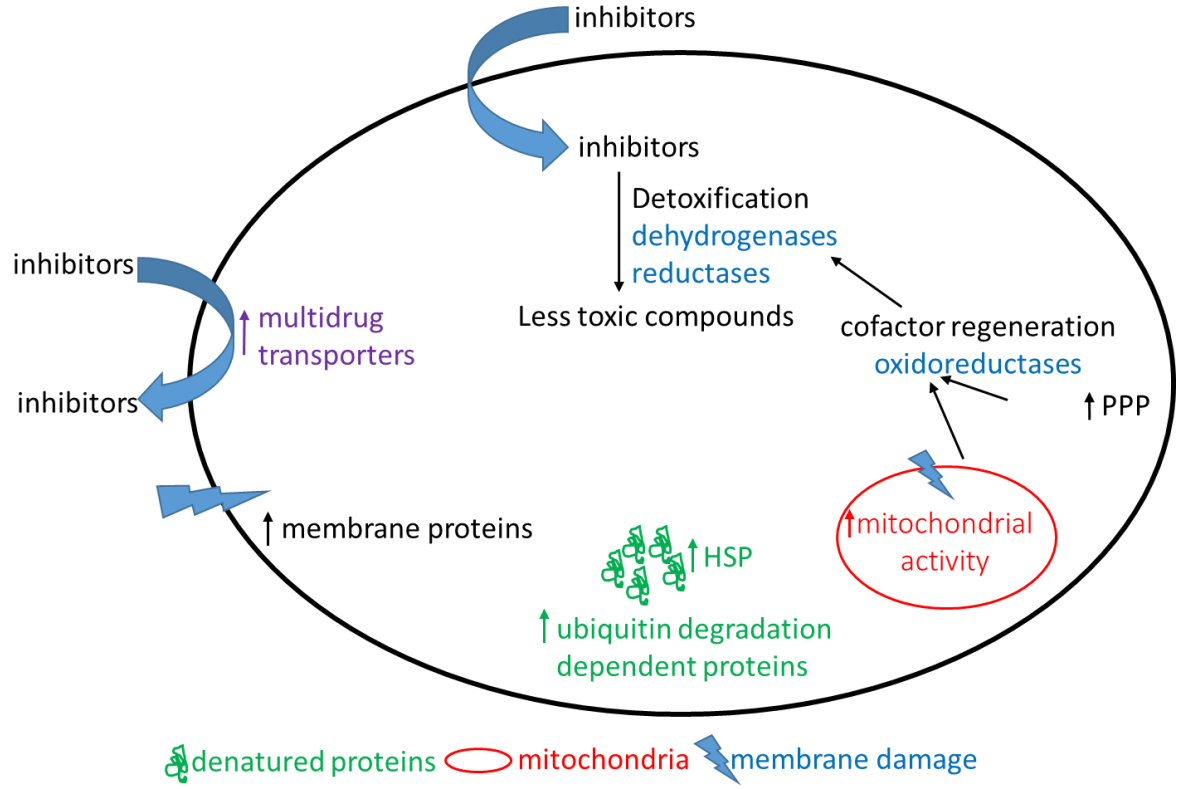

Figure 6: A schematic representation of the suggested mechanisms elicited by *S. cerevisiae* strain YI30 when fermenting in the presence of a synthetic inhibitory cocktail. The most likely inhibitor tolerance mechanism employed by this strain is presumably the detoxification of the inhibitors by converting it to less toxic compounds. Detoxification requires NADH/NADPH cofactors that are generated by oxidoreductases as well as by directing the central metabolism towards the TCA cycle and the PPP. Multidrug transporters may also be utilised to export the inhibitors out of the cell, thereby decreasing their effectiveness. Inhibitory compounds affect membrane integrity including the plasma membrane and the mitochondrial membrane, which affect the membrane and mitochondrial function. In addition, inhibitors have a denaturing effect on proteins, this could elicit the activity of heat shock proteins to assist with protein folding and guiding of the denatured proteins to the proteasome for degradation via the ubiquitin-proteasome pathway.

The first mechanism most probably relies on the detoxification of the inhibitory compounds to less toxic compounds. The various dehydrogenases and reductases required for the detoxification process need several cofactors, including NADH/NADPH. These cofactors are generated by oxidoreductases and by directing the central metabolism towards the PPP, facilitating the resupply of cofactors. A second mechanism that is utilised by the inhibitor tolerant YI30 is presumably the use of multidrug transporters to export the inhibitory compounds out of the cell, thereby decreasing their effectiveness. Inhibitory compounds also affect membrane integrity, compromising the plasma membrane and the mitochondrial membrane. This leads to a decrease in mitochondrial function and increases expression of genes involved in mitochondrial function. In addition, inhibitors have a denaturing effect on proteins, which elicit the activity of heat shock proteins that either assist with protein folding or guide the denatured proteins to the proteasome for degradation via the ubiquitin-proteasome pathway.

CONCLUSION

The environmental stress response is indicative of *S. cerevisiae's* ability to adapt to different environmental conditions. The study provides insight into the complexity of temperature and inhibitor tolerance and the integrated approaches the cell undertake to survive the industrial and environmental stresses. Both the natural *S. cerevisiae* strains YI13 and YI30, employ several mechanism to survive extreme environmental conditions, either by adapting metabolic processes to maintain cell viability or by eliciting various stress responses to combat and/or survive these harsh environments. The various survival mechanisms suggested in the inhibitor tolerant strain, YI30, are in line with those observed for industrial and genetically modified *S. cerevisiae* strains. It is suggested that the inhibitor tolerant YI30 strain responds to inhibitor stress by upregulating detoxification processes with a concomitant upregulation of the oxidative stress response to maintain the redox homeostasis of the cell. Furthermore, inhibitory compounds were probably removed using multidrug transporters.

The natural thermotolerant strain YI13 used in this study supports the neutral model, which proposes that natural *S. cerevisiae* strains are not adapted to a specific niche, but are able to survive due to a strong core response and cross-tolerance to various stress conditions (Goddard and Greig 2015). The thermotolerant strain YI13 experienced

oxidative stress probably due to an increase in ROS when the fermentation temperature is increased. This led to an upregulation of the oxidative stress response to maintain the redox potential within the cell. In addition, amino acid biosynthesis and ribosome biogenesis were upregulated presumably to increase the production of proteins irreversibly damaged by the ROS activity.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS

Saccharomyces cerevisiae serves as platform to elucidate biological processes in eukaryotic systems to expand the fundamental understanding of these processes, as well as applying these processes to assist in human development. *Saccharomyces cerevisiae* is used for the production of essential products including food, feed, and nutrients, addressing basic needs including health and disease management, and for the production of value-added products such as pharmaceuticals and non-essential commodity products (Foury 1997; Mustacchi et al. 2006; Johnson and Echavarri-Erasun 2011; Walker and Stewart 2016). The complex integrated functional networks displayed by its simplistic eukaryotic organisation allows colonisation of various environmental niches with extreme physical parameters, including high osmolarity, wide pH range, high temperatures and environments containing toxic compounds. This adaptability of *S. cerevisiae* is due to its genetic and phenotypic diversity and the genetic interaction between the organism and the environment (Mackay et al. 2009; Hittinger 2013; Marsit and Dequin 2015).

Natural *S. cerevisiae* strains exhibit a greater level of genetic and phenotypic diversity than observed in laboratory and industrial strains (Cubillos 2016). The diversity of these strains is associated with both the environment and geographical location of these strains (Clowers et al. 2015), and different environments therefore allow for the isolation of natural strains that represent unique phenotypes. In addition, the natural diversity of *S. cerevisiae* strains from South Africa has not been studied extensively and therefore provides a unique resource for obtaining diverse strains. Furthermore, hybridisation studies have been implicated in the generation of genetic diversity in *S. cerevisiae*, providing a research tool for enhancing genetic and thus phenotypic diversity (Pulvirenti et al. 2002; Pérez-Través et al. 2012).

Phenotypic diversity is a polygenic trait requiring the interaction of several genes, and differences in transcript abundance play an important role in phenotypic diversity (Mackay et al. 2009; Salinas et al. 2016). Gene expression variation is therefore an effective tool to determine the molecular mechanisms responsible for a specific

phenotypic characteristic. RNA-sequencing provides a useful platform to quantify transcripts and thus elucidate these molecular mechanisms.

6.1 NATURAL *SACCHAROMYCES CEREVISIAE* STRAINS AS MICROBIAL HOST

The environmental and geographical differences observed in the different wine regions of the Western Cape of South Africa provide a range of unique locations for assessing the diversity of *S. cerevisiae* strains (Robinson 2006; Stevenson 2005). *Saccharomyces cerevisiae* strains collected from these areas were evaluated for their phenotypic diversity with specific phenotypes, including those required for the production of cellulosic bioethanol such as temperature, osmo-, pH, ethanol and inhibitor tolerance (Klinke et al. 2004; Kumar and Sharma 2017). Several strains with increased phenotypic tolerance were identified (discussed in Chapter 3). Although not definitive, a general association was observed between the phenotype and the geographical location of the strains. Strains collected from the warmer inland regions with mineral rich and organic soils displayed a higher degree of sensitivity to the range of parameters assessed, whereas strains collected from the Cape Coastal area represented by average temperatures and acidic soils, displayed a greater level of tolerant phenotypes with regards to the parameters tested. The South Coastal regions that are associated with cooler temperatures and mineral rich soils, harboured strains with an increased glucose fermentation capacity.

The strains displayed similar temperature-, pH and salt tolerance with 16 % and 7 % of them being tolerant against a 25 % synthetic inhibitor cocktail and 20 % (v/v) ethanol concentration, respectively. Strain YI13 displayed the highest temperature tolerance, capable of growth at 45 °C compared to 42 °C for the majority of the strains, together with intermediate tolerance against 15 % v/v ethanol and the 15 % inhibitor cocktail. In addition, two inhibitor tolerant strains, HR4 and YI30, capable of maintaining ethanol productivity in the presence of a 25 % inhibitory cocktail during aerobic and anaerobic conditions, respectively, were identified. Although general trends could be observed that associated specific regions with specific traits, the phenotypic diversity displayed by a particular strain is a unique trait, and dependent on the specific strain.

6.2 PHENOTYPIC IMPROVEMENT THROUGH ARTIFICIAL MATING

Hybridisation is an effective method to generate genetic diversity and thereby enhance phenotypic characteristics or allow for the generation of novel characteristics (Krogerus et al. 2017). Interspecies and intraspecies hybridisation permit the generation of unique phenotypes by allowing the combination of different parental genotypes where the progeny may display an intermediate phenotype, heterosis or a diminished phenotype (Da Silva et al. 2015). Although several diverse progenies were generated, no hybrid strains displaying a combined karyotype that represents both parental profiles were obtained. This suggests that intertetrade mating was the main mechanism for the generation of progeny strains. This could be due to the experimental design as only progeny strains that displayed an altered phenotype for the characteristics assessed (pH, temperature, salt, inhibitor cocktail and ethanol) were evaluated.

Several unique karyotypes were observed that were probably generated through chromosomal rearrangement during recombination of identical genotypes. In addition, haplo-selfing has been implicated as a core mechanism that permits genome renewal (Pretorius 2000). This allowed for the generation of phenotypes that varied from those displayed by the parental strains. In addition, homozygosity may have permitted unique phenotypes to be displayed through the manifestation of recessive traits. In this study, haplo-selfing and intratetrade mating were enhanced, thus allowing the persistence of a particular parental genotype, specifically the YI30 parental genome.

It has been shown that sporulation favours intratetrade mating to maintain essential genes required for adaptation to adverse environmental conditions (Knop 2006). This also could explain why the production of multi-tolerant strains was not observed, except for one strain (V3/YI30#6) that displayed extreme osmo-, temperature and inhibitor tolerance as well as high pH and ethanol tolerance (Chapter 4). In addition, the high energy demand that is required for robustness could also hinder the generation of multi-tolerant strains as an excessive energy demand is detrimental to cell viability, thus decreasing the population size of these isolates and thus lowering the possibility of isolating this minority population (De Visser et al. 2003; Maltsev et al. 2005).

6.3 CELLULAR MECHANISMS ASSOCIATED WITH TEMPERATURE TOLERANCE

Tolerances to environmental stresses are polygenic traits that require the interaction of a diverse range of genes that are often regulated on a transcriptional level (Salinas et al. 2016). Transcriptomic analysis therefore provides an appropriate platform to determine the molecular mechanisms that are associated with a specific phenotypic characteristic. Temperature tolerance requires a myriad of mechanisms due to the damaging effects an increase in temperature has on both structural and functional cellular components. An integrated network of stress mechanisms is therefore required to combat the damage inflicted by an increase in temperature and to maintain cell viability and fermentation capacity during industrial processes. Several studies investigated the mechanisms responsible for temperature tolerance and a range of stress responses have been identified that confer tolerance in industrial and genetically engineered strains (Strassburg et al. 2010; Walther et al. 2010; Ismail et al. 2013). However, the transcriptional landscape implicated in temperature tolerance of natural strains has not been studied to the same extent.

In this study, the transcriptomic profile of a natural *S. cerevisiae* strain YI13 was investigated, specifically in relation to early induction during ethanol production. In contrast to other studies with the aim to elucidate the temperature tolerance mechanisms, the heat shock response was not the major stress response elicited in this strain. It could be that this strain does not elicit this response as the main temperature tolerance mechanism, or that the proteins of this strain are more resistant to temperature damage. Alternatively, it could be due to the experimental set-up that specifically evaluated fermentation capacity and thus the transcriptome was assessed at the early induction phase, possibly before heat could cause protein damage to elicit a heat shock response. In addition, the experimental and control conditions evaluated the same temperature tolerant strain. This strain is able to grow at a maximum of 45 °C and therefore displays a native ability to withstand high temperatures, and a fermentation temperature of 37 °C may be too low to elicit a heat shock response in this particular strain.

The major response observed in strain YI13 was associated with ribosome biogenesis and amino acid biosynthesis. It is postulated that this could be in response to the oxidative damage to proteins and protein-complexes that required an enhanced

generation of ribosomes as the workhorse, as well as amino acids as the building blocks for protein biosynthesis. The increased protein damage is possibly due to an increase in ROS that were generated in response to an increase in temperature. Since fermentation capacity was specifically assessed and not cell growth, the increased temperature may have been exacerbated by the metabolic activity of the cells. ROS cause irreversible damage to sulfhydryl proteins specifically that cannot be effectively managed by the HSR chaperone activity, hence protein activity had to be maintained via an upregulation in protein synthesis.

6.4 CELLULAR MECHANISMS IMPLICATED IN CELLULOSIC INHIBITOR TOLERANCE

As indicated in the abovementioned temperature tolerance mechanisms, phenotypic characteristics such as inhibitor tolerance mechanisms rely on a complex network of integrated mechanisms to maintain cell viability and fermentation capacity when exposed to inhibitory compounds. Inhibitor tolerance in industrial strains has received much attention in the drive towards second-generation bioethanol production. Several molecular mechanisms have been implicated and a multitude of genes have been identified that confer resistance to cellulosic inhibitory compounds (Sasano et al. 2012; Kim and Hahn 2013; Ma et al. 2015; Swinnen et al. 2017). The majority of these studies focused on single inhibitors and the performance of industrial or genetically engineered strains with only a few investigating the effects of an inhibitory cocktail (Li and Yuan 2010; Ma and Liu 2010; Bajwa et al. 2013; Zhou et al. 2014; Lee et al. 2015; Chen et al. 2016; Thompson et al. 2016; Ibáñez et al. 2017). In addition, the molecular mechanisms employed by natural strains have not been studied and thus the question remains as to whether similar mechanisms are employed by natural strains to maintain fermentation capacity in the presence of inhibitory compounds. In our study, oxidative stress response was the main mechanism activated to combat inhibitor exposure. The transcriptional data for strain YI30 suggest an active detoxification mechanism to convert the inhibitory compounds to less toxic compounds. The detoxification process requires the activity of several dehydrogenases and reductases, which in turn require cofactors. This causes an upregulation in the oxidative stress response in order to generate the necessary cofactors as well as ATP to maintain the energy requirements for this response and for maintaining cellular processes.

From the data presented in this study, the following can be concluded:

- there is a broad association between the geographical location, the environment and the phenotypic diversity displayed by natural *S. cerevisiae* strains
- individual natural *S. cerevisiae* isolates display unique characteristics that do not necessarily associate with the general isolates or the specific niche of the isolate
- spore-to-spore mating promotes intratetrade mating
- intertetrade mating does allow for the generation of unique phenotypes
- generation of specific phenotypic characteristics cannot be predicted
- there is a limit in the degree of phenotypic diversity displayed by a specific *S. cerevisiae* strain
- the main temperature tolerance mechanism displayed by strain YI13 related to maintaining protein activity via the upregulation of ribosome biogenesis and amino acid biosynthesis, as well as maintenance of the oxidative stress response - possibly to combat ROS activity
- additional temperature tolerant responses were observed that related to membrane damage that affect plasma membranes, including mitochondrial and nuclear membranes resulting in an upregulation in mitochondrial and DNA repair proteins to combat mitochondrial and DNA damage, as well sterol production to maintain cell wall integrity
- the main inhibitor tolerance mechanism displayed by strain YI30 presumably related to the detoxification of the inhibitory compounds via the upregulation of dehydrogenase and reductase activity, with a concomitant upregulation of the oxidative stress response for the generation of the required cofactors and energy production, as well as an increase in multidrug transporters for the removal of inhibitory compounds
- other inhibitor tolerance responses related to membrane damage that affected plasma membranes (including mitochondrial membranes) and thus resulted in an upregulation in mitochondrial proteins
- furthermore, an increase in the HSR was observed possibly to maintain the integrity of proteins damaged by the inhibitory compounds
- evaluation of natural strains to obtain unique phenotypes allows for the isolation of strains that display novel phenotypes that can be exploited for the production of second-generation bioethanol

- the unique characteristics displayed by natural *S. cerevisiae* strains can be exploited to elucidate the biological processes and molecular mechanisms responsible for these novel characteristics

6.5 LIMITATIONS AND RECOMMENDATIONS

In Chapter 3, similar environments were evaluated that did allow for the identification of strain diversity. Assessing a wider range of unique environments may allow for an increased phenotypic diversity and thus the identification of more robust strains. In addition, combining several stress factors during the screening process may have allowed for the identification of multi-tolerant natural strains.

No true hybrid strains that displayed the combined karyotype of the two parental strains were obtained in this study and there is no data to suggest that they were obtained but excluded due to the selection criteria. Hybrid strains should therefore be assessed to elucidate whether an increased multi-tolerance is attainable. Furthermore, screening individual spores for the required phenotype before mating may have allowed for the generation of multi-tolerant hybrid strains.

Both the phenotypic characterisation and transcriptomic assessment were performed in a carbon-rich medium that provided sufficient carbon for energy production when the strains were exposed to environmental stress. Assessing these parameters in a carbon-limited environment may allow for the identification of additional strains and mechanisms that allow environmental tolerance when energy generation is limited. This also applies to the availability of oxygen. *Saccharomyces cerevisiae* strain HR4 for instance, displayed inhibitor tolerance in the presence of oxygen and this strain can be used to elucidate the specific molecular mechanisms involved in this phenotype.

6.6 FUTURE STUDIES

Natural strains isolated from a wider range of environments and geographic locations should be evaluated to identify unique strains that could be utilised in fundamental, industrial and biotechnological applications. Genomic comparison of the parental and progeny strains that displayed heterosis and/or unique phenotypes could provide insight into the mechanisms involved in these phenotypes. Transcriptomic analysis was utilised to elucidate the molecular mechanisms associated with adaptation to a specific

environment. These observations should be experimentally evaluated to conclusively determine whether they are responsible for the phenotypic characteristics displayed by the specific strain.

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ADDENDUM

MATING OF NATURAL *SACCHAROMYCES CEREVISIAE* STRAINS FOR IMPROVED GLUCOSE FERMENTATION AND LIGNOCELLULOSIC INHIBITOR

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ORIGINAL ARTICLE

Mating of natural *Saccharomyces cerevisiae* strains for improved glucose fermentation and lignocellulosic inhibitor tolerance

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Abstract Natural *Saccharomyces cerevisiae* isolates from vineyards in the Western Cape, South Africa were evaluated for ethanol production in industrial conditions associated with the production of second-generation biofuels. The strains displayed high phenotypic diversity including the ability to grow at 45 °C and in the presence of 20% (*v*/*v*) ethanol, strain YI13. Strains HR4 and YI30 were inhibitor-tolerant under aerobic and oxygen-limited conditions, respectively. Spore-to-spore hybridization generated progeny that displayed heterosis, including increased ethanol productivity and improved growth in the presence of a synthetic inhibitor cocktail. Hybrid strains HR4/YI30#6 and V3/YI30#6 were able to grow at a high salt concentration (2 mol/L NaCl) with V3/YI30#6 also able to grow at a high temperature (45 °C). Strains HR4/YI30#1 and #3 were inhibitor-tolerant, with strain HR4/YI30#3 having similar productivity (0.36 ± 0.0036 g/L per h) as the superior parental strain, YI30 (0.35 ± 0.0058 g/L per h). This study indicates that natural *S. cerevisiae* strains display phenotypic variation and heterosis can be achieved through spore-to-spore hybridization. Several of the phenotypes (temperature-, osmo-, and inhibitor tolerance) displayed by both the natural strains and the generated progeny were at the maximum conditions reported for *S. cerevisiae* strains.

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Introduction

Although the production of first-generation biofuels from predominantly maize and sugarcane has been successful in some countries (e.g., USA and Brazil), the application is limited and detrimental in developing countries where food security rather than energy security is a threat to human survival (van Zyl et al. 2011). These countries need to invest in alternative feedstocks for sustainable biofuel production. Cellulosic biomass has been identified as an abundant and renewable feedstock (Lynd 1996). However, cellulosic biofuel production requires pretreatment of the lignocellulosic feedstocks to render the polysaccharides accessible for saccharification and fermentation.

Enzymatic pretreatment requires the addition of exogenous enzymes or heterologous production of enzymes for the conversion of alternative feedstocks. Increased enzyme activity is obtained at elevated temperatures, subsequently lowering the amount of enzymes needed for substrate conversion (Viikari et al. 2007). The fermentation process is an exothermic reaction, reaching higher temperatures than the optimal temperature of the microbial organism (Nevoigt 2008). In order to minimize contamination, optimize enzyme activity, and simplify product recovery, current biofuel production is performed at elevated temperatures, thus lowering production costs (Benjaphokee et al. 2012; Nevoigt 2008). Therefore, use of temperature-tolerant strains provide an advantage when choosing a microbial host for the cost-effective production of second-generation biofuels.

Chemical pretreatment increases the osmolarity of the environment due to the addition of inorganic compounds (acids, alkalis, and ionic solvents) (Jönsson and Martín 2016). The increase in the osmotic pressure affects cell viability and ethanol production (Jönsson et al. 2013; Jönsson and Martín 2016; Balakumar and Arasaratnam 2012). Hence, the use of osmo-tolerant strains is advantageous when producing second-generation biofuels. The presence of these salts concomitantly affect the pH of the hydrolysate, thus pH tolerant strains are beneficial when selecting a strain for use in the production of second-generation biofuels.

Pretreatment leads to the formation of various lignocellulosic hydrolysate compounds that inhibit microbial fermentation (Larsson et al. 1999; Larsson et al. 2000). The type of inhibitory compounds generated during the pretreatment process depends on both the specific pretreatment employed as well as the type of feedstock used (Jönsson and Martín 2016). The inhibitory products can be divided into three major groups: aromatic and phenolic compounds (from lignin), aliphatic and uronic acids (from hemicellulose), and aliphatic (from hemicellulose) and furan aldehydes (from degradation of sugars). The inhibitory compounds affect the fermentation process (reduce ethanol yield and productivity), the fermenting organism (reduce vitality and growth), as well as the cellulolytic enzyme activity (product inhibition and toxicity) (Almeida et al. 2007; Jönsson and Martín 2016; Mhlongo et al. 2015). Inhibitor-tolerant organisms are therefore an attractive alternative to ferment the hydrolysate produced after the pretreatment procedure. The pursuit for alternative energy sources and the success of the production of first-generation biofuels has brought renewed efforts into identifying robust organisms that can be used to produce second-generation biofuels and various other biocommodities (Lynd et al. 1999; Lynd et al. 2008).

Saccharomyces cerevisiae is the preferred yeast species for use in the majority of industrial processes and has been exploited in the production of alcohol since 6000 BC (Walker 1998; Robinson 1994). The robust nature of this species allows for survival during various industrial processes, and due to its wide use, it has been extensively studied (Walker 1998; Zaldivar et al. 2001). *S. cerevisiae* laboratory strains have a defined genetic structure and are isogenic, heterothallic, and usually haploid. Natural *S. cerevisiae* strains, however, are genetically diverse, homothallic, diploid, polyploid or aneuploid, and prototrophic (Walker 1998; Randez-Gil et al. 1999).

Yeast strain diversity varies according to geographical region and environmental conditions. Therefore, natural occurring *S. cerevisiae* strains isolated from different environments differ significantly in phenotypic characteristics (Kvitek et al. 2008; Liti et al. 2009; Camarasa et al. 2011) and resistance to inhibitory compounds (Favaro et al. 2013). Currently, the most successful industrial bioethanol-producing strains are *S. cerevisiae* strains isolated from industries producing first-generation bioethanol (Basso et al. 2008; Mukherjee et al. 2014). It is, therefore, feasible, that by exploiting the environmental diversity, natural occurring strains may be obtained that can be utilized to produce second-generation biofuels on an industrial scale.

Classic genetics is a useful tool to understand the mechanisms of phenotypic variation. Natural inter- and intraspecies hybridization may generate hybrid strains with improved, superior, or novel characteristics. Breeding strategies have been used to improve industrial strains with complex traits, allowing the generation of hybrids that outperform the parental strains (Marullo et al. 2009; Meersman et al. 2015; Benjaphokee et al. 2012). Fusion between genetically different spores may lead to the optimization of complex phenotypes or the combining of superior traits, whereas haploselfing may allow desirable recessive traits to be uncovered, giving rise to novel phenotypes (Marullo et al. 2009; Meersman et al. 2015; Benjaphokee et al. 2012; Steensels et al. 2014).

The current study explores the natural diversity of *S. cerevisiae* vineyard strains within the ecological and geographical diverse Western Cape region of South Africa. Natural *S. cerevisiae* strains were evaluated in different environments (temperatures, pH, salt, inhibitory compounds, and ethanol concentrations). The ability to utilize glucose aerobically and in a limited oxygen environment in the presence of ethanol and a synthetic lignocellulosic inhibitor cocktail were assessed. *S. cerevisiae* strains that performed well during the initial screen were selected for spore-to-spore mating, where after the progeny were evaluated for ethanol production in a limited oxygen environment in the presence and absence of inhibitory lignocellulosic compounds. The strains generated during this study displayed various enhanced tolerant phenotypes in comparison to the parental strains.

Materials and methods

Strains

Indigenous natural *S. cerevisiae* strains from within the Western Cape in South Africa were obtained from ARC Infruitec-Nietvoorbij. Strains were collected from vineyards from diverse environmental and geographical regions (Cape Winelands, Cape Peninsula, and Overberg representing cool coastal and warmer inland regions) as part of the ARC Infruitec-Nietvoorbij breeding and evaluation program (van der Westhuizen et al. 2000b; van der Westhuizen et al. 2000a; Khan et al. 2000). Briefly, grapes were collected and allowed to ferment spontaneously, and *S. cerevisiae* and non-*Saccharomyces* strains were isolated at the end of the fermentation process. Fifty-six *S. cerevisiae* strains were randomly

selected and propagated in YPD media (1% yeast extract, 2% peptone, and 2% glucose), and glycerol stocks (30% *v*/*v*) were prepared. *S. cerevisiae* strain MH1000, a robust homothallic, industrial distillery strain, was used as a reference strain (Viktor et al. 2013). All experiments were performed in biological triplicates.

Phenotypic characterization

Spot assays were performed as follows: Strains were grown overnight at 30 °C in YPD medium. Serial dilutions (10^{-1}, 10^{-2}, and 10^{-3}) of overnight cultures (i.e., stationary phase) were prepared and spot inoculated on the relevant agar plates. Plates were incubated at 37 °C and monitored daily for 3 days. Plates assessing growth in environments that contained volatile compounds (ethanol and lignocellulosic inhibitory compounds) were sealed with parafilm in order to minimize evaporation. These plates were monitored for growth on day 7 in order to determine whether evaporation played a role during the experimental procedure. Temperature tolerance was investigated by spot inoculating onto YPD plates with incubation at different temperatures (26, 30, 37, 40, 42, and 45 °C). Strains were assessed for ethanol- and osmotolerance by spot inoculating onto YPD plates containing 10, 15, and 20% (*v*/*v*) ethanol and 0.5, 1.0, 1.5, and 2.0-mol/L NaCl, respectively with incubation at 30 °C. Inhibitor tolerance was evaluated by spot inoculating onto YPD plates containing a synthetic inhibitor cocktail (25, 50, and 75%). The synthetic inhibitor cocktail contained at least one representative of each of the major inhibitory compound groups found in various pretreated feedstocks, at inhibitory concentrations (Martín and Jönsson 2003; Jönsson and Martín 2016). The 25% inhibitor cocktail composition was as follows: 0.88g/L formic acid, 1.13g/L acetic acid, 0.73g/L furfural, 0.88g/L HMF, 0.038g/L cinnamic acid, and 0.45g/L coniferyl aldehyde (Martín and Jönsson 2003). pH Tolerance was evaluated by incubating the strains at 30 °C in 1mol/L citrate-buffered synthetic complete (SC) medium (1.7g/L YNB, 5g/L $(NH_4)_2SO_4$, 20g/L glucose) with an initial pH ranging from pH 2–11. Cell growth was evaluated spectrophotometrically ($A_{600\ nm}$) after incubation at 30 °C. The pH at the end of the incubation period did not differ significantly from the initial pH. The physiological tolerance was scored as follows: where multiple conditions for a single environment was tested (temperature, ethanol, pH, and osmolarity), a score of 6 indicates growth after 3 days at the highest dilution for all the conditions tested and a score of zero indicates no growth after 3 days for the undiluted sample for all the conditions tested. Where a single condition for an environment was tested (inhibitor), a score of six indicates growth after 1 day at the highest dilution and a score of zero no growth after 3 days for the undiluted sample. Strains were evaluated for sporulation by inoculating overnight cultures onto sporulation plates (1% potassium agar) with 1-week incubation at room temperature. Wet mounts were prepared and microscopically viewed for the formation of ascospores.

Glucose utilization

Strains were screened for the ability to ferment glucose in a limited oxygen environment. Static fermentation experiments were performed at 30 °C as described by Favaro et al. (2013). Briefly, strains were grown overnight in must nutrient synthetic (MNS) minimal medium (Delfini 1995) with 20% (*w*/*v*; 200 g/L) glucose. Overnight cultures were inoculated (7.5×10^4 cells per mL) into 110mL serum bottles containing 100mL MNS medium. Reaction vessels were sealed with rubber stoppers and each vessel was equipped with a syringe needle plugged with cotton wool for the removal of carbon dioxide (CO_2) produced during the fermentation reaction. Glucose utilization was evaluated in the presence and absence of a synthetic 25% lignocellulosic inhibitor cocktail. Growth was monitored daily by measuring weight loss in relation to CO_2 production. Results were reported (using a conversion factor of 2.118) as grams of glucose utilized (Favaro et al. 2013). Since some of the glucose is converted to glycerol (g/mol ratio ~ 0.072) with the remainder of the glucose converted to CO_2 (g/mol ratio ~ 2.048), a total conversion coefficient of 2.118 was empirically determined (Delfini 1995; Delfini and Formica 2001). Fermentation reactions performed in serum bottle were regarded as oxygen-limited due to the experimental set-up (limited headspace with a reaction volume of 91% of the working volume; crimp sealed with a rubber stopper with no or low agitation (150 rpm). Fermentation experiments were conducted at pH 3.5, as an acidic environment is often associated with pretreated feedstocks and a temperature of 30 °C as this is the optimal growth temperature of the strains.

Aerobic growth characterization

Strains that performed well during the glucose utilization experiments and displayed tolerant phenotypes were selected for further investigation. These strains were deposited in the Plant Protection Research Institute (PPRI) database in Pretoria, South Africa. This database forms part of the World Federation for *Culture Collections* (WFCC) a registered living culture collection; a division of the National Collection of Fungi, under the custodianship of the ARC. The respective identification numbers for these strains are HR4: 21385, V3: 21381, YI13: 21378, and YI30: 21386. Shake flasks (1 L) containing 300mL SC medium were inoculated (10% *v*/*v*) with overnight cultures and grown for 24 h on a rotary shaker at 200 rpm at 30 °C with sampling every 3 h. Cell growth was evaluated spectrophotometrically ($A_{600\ nm}$). Glucose utilization and ethanol production were monitored by high-pressure liquid chromatography (HPLC), using a Surveyor Plus liquid

chromatograph (Thermo Scientific) consisting of an LC pump, autosampler, and refractive index detector. Compounds were separated on a Rezex RHM Monosaccharide 7.8 × 300mm column (00H0132-K0, Phenomenex) at 60 °C with 5-mmol/L H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min. These parameters were also investigated in the presence of 10% (*v*/*v*) ethanol and a synthetic 25% inhibitor cocktail. Strains were grown for 3 days with sampling every 24 h. An initial ethanol concentration of 10% ethanol was used to identify strains capable of ethanol production in the presence of existing ethanol in order to evaluate ethanol tolerance. Fermentation reactions performed in shake flasks were regarded as aerobic due to the experimental set-up (ample headspace with a reaction volume of 30% of the working volume and agitation at 200 rpm).

Ethanol production

Ethanol production of selected strains was assessed under various conditions including osmotolerance (20% *v*/*v* glucose), ethanol tolerance (10% *v*/*v*), and inhibitor tolerance (25%). Fermentation reactions were performed as described in the glucose utilization section with minor modifications. Each fermentation vessel was equipped with a magnetic stirrer bar and two syringe needles; one plugged with cotton wool for the removal of CO_2 and one connected to a 2mL syringe for sampling. The fermentation vessels were incubated on magnetic stirrers at 30 °C for 14 days. Fermentations were monitored daily by measuring cell growth spectrophotometrically ($A_{600\ nm}$) and ethanol and glucose concentrations using HPLC. Ethanol yield (g/L) was determined from the slope of the curve of the fitted straight line obtained after plotting the ethanol produced (g/L) against the amount of glucose utilized (g/L). Ethanol productivity (g/L per h) was calculated as the maximum amount of ethanol (g/L) produced divided by the total time (hours) taken to produce the ethanol.

Hybrid generation

Spore-to-spore mating was performed between the four selected *S. cerevisiae* strains (HR4, V3, YI13, and YI30). Briefly, strains were allowed to sporulate on sporulation agar for 1 week at room temperature. Asci were suspended in 2mL sterile double distilled water (ddH_2O) and washed twice (centrifuged at 5000 rpm for 5 min and resuspended in 500μL ddH_2O). Asci were resuspended in 150μL lysis buffer (0.5mg/mL zymolase in 1mol/L glucitol) and incubated overnight at 30 °C. Spores were washed with 2mL ddH_2O and pellets resuspended in 500μL ddH_2O. The Singer MSM System 200 micromanipulator microscope (Singer Instruments, Somerset, England) was used to dissect the spores on YPD plates. Spores from different strains were allowed to mate and the diploids obtained were evaluated for the various criteria as mentioned previously.

Strain verification

To confirm the authenticity of the indigenous and the generated progeny, interdelta PCR using the delta 12–21 primer set (δ12; TCA ACA ATG GAA TCC CAA C and δ21; CAT CTT AAC ACC GTA TAT GA) (Legras and Karst 2003) as well as electrophoretic karyotyping (CHEF) was performed (Hoff 2012). Interdelta region PCR amplification: 50μL reaction volume containing 20ng yeast DNA, 10× reaction buffer (Southern Cross Biotechnologies PTY (LTD), 25mmol/L $MgCl_2$, 10 μmol/L of each oligonucleotide primer, 2.5 mmol/L of each dNTP, and 0.5U Super-Therm Taq polymerase (Southern Cross Biotechnologies PTY (LTD). PCRs were performed with a BioRad cycler using the following program: 4 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 48 °C, 90 s at 72 °C, and a final elongation step of 10 min at 72 °C. The selected strains were compared to a reference *S. cerevisiae* (CBS 1171) strain and the industrial *S. cerevisiae* strain, MH1000. Electrophoretic gels were run on a CHEF DRIII system (BioRad, USA) for 34 h at a constant voltage of 6 V/cm. The initial pulse duration was 30 s and the final pulse duration 215 s.

Results

Environmental conditions relevant in second-generation bioethanol production were evaluated. Host strains for second-generation ethanol production need to be ethanol-tolerant (due to product formation), temperature-tolerant (due to the exothermic fermentation process and high optimal temperature of cellulase enzymes, typically above 40 °C), osmo- and pH-tolerant (due to salts and acids used in the pretreatment process), and inhibitor-tolerant (due to the inhibitory compounds formed during pretreatment). The ability of natural *S. cerevisiae* strains to grow in these different environments was evaluated and compared to a robust industrial distillery strain, *S. cerevisiae* MH1000. The strains displayed a variety of phenotypes as indicated in Fig. 1.

The average annual environmental temperature of the regions where the strains were collected ranges between 16.5 and 23.3 °C, thus these strains were unlikely to be thermotolerant; however, a maximum growth temperature of 42 °C was observed for most of the strains with *S. cerevisiae* strain YI13 able to grow at 45 °C (Fig. 1). The genus *Saccharomyces* is classified as mesophiles, with *S. cerevisiae* the thermo-tolerant species with a maximum growth temperature of 45 °C (Salvadó et al. 2011) and an optimal growth and fermentation temperature of 30 °C (Lu et al. 2012). Though rare, growth at 45 °C is not unexpected for *S. cerevisiae* strains. Most of the natural *S. cerevisiae* strains were able to grow in the presence of 10% (*v*/*v*) ethanol; however, only a few strains were able to grow in the presence of 15–20% (*v*/*v*) ethanol (Fig. 1). None of the strains

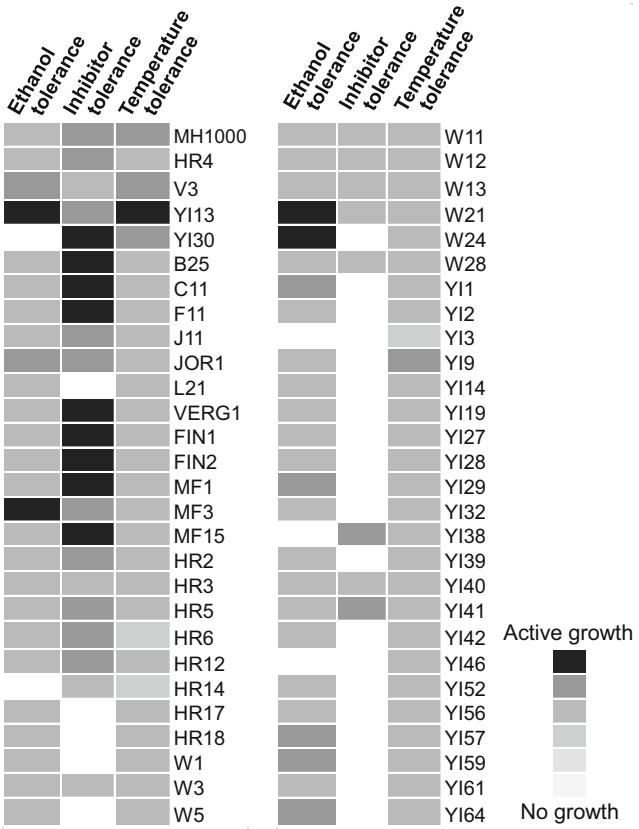

Fig. 1 Phenotypic diversity of natural strains. The viability of 56 natural strains to various environments (ethanol, inhibitor, temperature, pH, and salt) was compared to the industrial strain MH1000 (pH and salt data were omitted as the strains showed similar phenotypes for these environments). Each row represents a different strain and each column a specific environment. The phenotype tolerance was scored as follows: where multiple conditions for a single environment were tested (temperature and ethanol); a score of 6 indicates growth after 3 days at the highest dilution for all the conditions tested and a score of zero indicates no growth after 3 days for the undiluted sample for all the conditions tested). Where a single condition for an environment was tested (inhibitor), a score of 6 indicates growth within 1 day at the highest dilution and a score of zero no growth after 3 days for the undiluted sample) (adapted from Kvitek et al. 2008)

were able to grow in the presence of 50 and 75% of the inhibitor cocktail, with only a few strains able to grow in the presence of a 25% inhibitor cocktail (Fig. 1). All strains were able to grow at the pH range (pH 2–11) and salt concentrations (0.5–1.5 mol/L) tested (data not shown). The strains were evaluated for the ability to sporulate. All strains were able to sporulate and were therefore, most likely diploid.

The second aim of this study was to establish whether heterosis could be obtained via mating. The aim was to either enhance the phenotype by generating strains with improved phenotypes compared to the superior parent or by obtaining mid-parent heterosis. The physiological characteristic data in Fig. 1 was used to select four strains for further experimentation. YI13 was selected as a tolerant strain due to tolerance to all conditions tested, whereas V3 was selected as a mid-tolerant strain. HR4 was selected as a sensitive strain due to the ethanol and temperature sensitivity and YI30 was selected for its varied phenotype with inhibitor tolerance, mild temperature tolerance, and sensitivity towards ethanol.

Growth, ethanol production, ethanol tolerance, and inhibitor tolerance of the four selected strains were evaluated in aerobic (Fig. 2) and oxygen-limited (Fig. 3) environments. Although the strains were able to grow at 37 °C, ethanol production was lower than observed for fermentations performed at 30 °C (data not shown). Most of the strains were able to grow aerobically in the presence of 10% (*v*/*v*) ethanol (Fig. 2c); however, none of the strains was able to ferment glucose in the presence of an initial ethanol concentration of 10% (*v*/*v*) (Fig. 2d). *S. cerevisiae* strain, HR4, was able to grow aerobically in the conditions tested (Fig. 2c and e) and ferment in the presence of 25% inhibitor cocktail (Fig. 2f).

Ethanol and inhibitor stress under oxygen-limited conditions had a similar effect on biomass production as none of the strains showed considerable differences (p value below 0.05 for single-factor ANOVA, Microsoft Excel data analysis tool pack) in the ability to grow in oxygen-limited conditions, in the presence of either 10% (*v*/*v*) ethanol or the inhibitor cocktail (Fig. 3c and e). None of the strains was able to ferment in the presence of an initial ethanol concentration (10% *v*/*v*) (Fig. 3d). Although the strains showed restricted growth in oxygen-limited conditions in the presence of the inhibitor cocktail (Fig. 3e), strain YI30 was inhibitor-tolerant with the ability to ferment in the presence of an inhibitor cocktail (Fig. 3f). This is indicative of the stress response where cells enter into the stationary phase while maintaining metabolic activity.

Spore-to-spore mating of the four selected strains produced viable progeny for all combinations except HR4/V3 (possibly due to genomic instability leading to the generation of non-viable progeny). The physiological characteristics of the generated progeny strains are summarized in Fig. 4. Forty-three percent of the generated strains displayed improved growth (growth at 10^{-3} dilution after 24 h) in the presence of the 25% synthetic inhibitor cocktail, compared to the parental strains (growth at 10^{-2} dilution after 48 h). Similarly, improved growth in an acidic (pH 2) environment was observed for strains HR4/YI13#16, HR4/YI30#2, V3/YI13#3, and YI13/YI30#1–6. Strains V3/YI30#6 and HR4YI30#6 also indicated enhanced growth (growth at 10^{-3} dilution after 24 h) in a high salt concentration (2mol/L NaCl) environment, in comparison to the parental strains (growth at 10^{-1} dilution after 72 h). Contrary to the parental strains, strain V3/YI30#6 was able to grow at 45 °C and strains HR4/YI30#4 and #6 were able to grow in the presence of 20% (*v*/*v*) ethanol.

The progeny strains were evaluated for growth and ethanol production in an oxygen-limited environment. Growth for most of the progeny did not differ significantly from that of the parental strains with the progeny produced from the HR4/

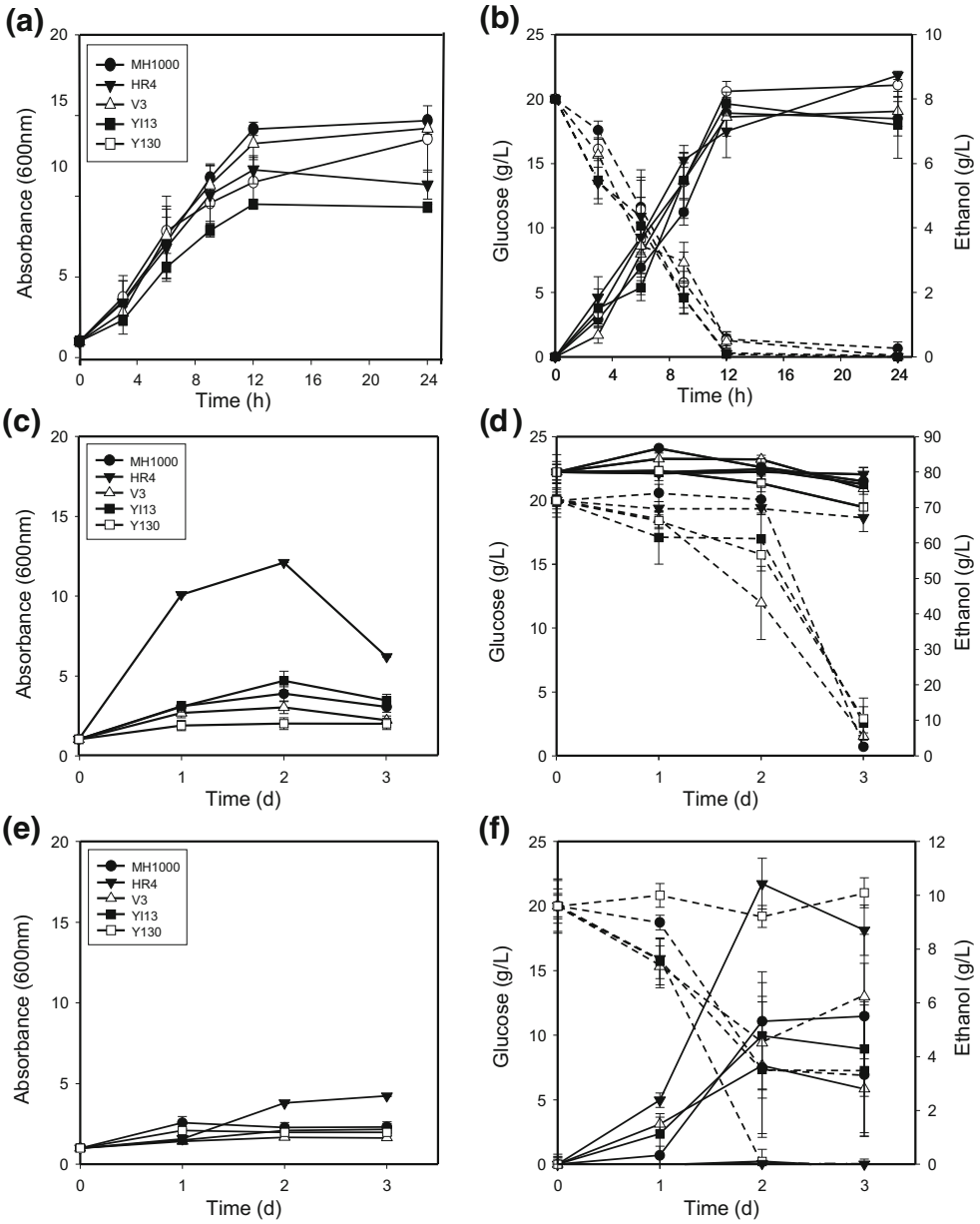

Fig. 2 Aerobic fermentations of parental strains in SC medium. Cell growth [depicted in the right side panel (**a**, **c**, and **e**)] was measured spectrophotometrically at $A_{600\ nm}$. Growth in (**a**) SC medium, (**c**) SC medium with 10% ethanol, and (**e**) SC medium with 25% inhibitor cocktail is indicated. Ethanol production [depicted in the left side panel (**b**, **d**, and **f**)] was monitored by HPLC. Residual glucose is indicated by the broken line and ethanol production by the solid line. Fermentations performed in (**b**) SC medium, (**d**) SC medium with 10% ethanol, and (**f**) SC medium with 25% inhibitor cocktail are indicated. Data series and error bars represent the mean values and the standard error of biological triplicates. Data points where error bars are not visible are due to low variation in biological triplicates

YI13 mating showing reduced growth (data not shown). The ethanol yield (as a percentage of the maximum theoretical yield, where the theoretical maximum ethanol yield (100%) is 0.51g/L ethanol per 1 g of glucose) and productivity (g/L per h) for the generated progeny are depicted in Fig. 5. In general, mating experiments with strain YI30 as one of the parental strains resulted in progeny with increased ethanol productivity, whereas the opposite is true for mating with strain YI13 as one of the parental strains (Fig. 5).

Two strains, HR4/YI30#1 and HR4/YI30#3, were able to ferment in the presence of a 25% inhibitor cocktail (Fig. 6b). Strain HR4/YI30#3 produced similar amounts of ethanol in the absence (Fig. 6a) and presence (Fig. 6b) of the inhibitor cocktail. This strain also had similar productivity (0.36 ± 0.0036 g/L per h) as the superior parental strain YI30 (0.35 ± 0.0058 g/L per h). The growth of this strain in the presence of the inhibitor cocktail did not differ to that of the parental strains (data not shown).

CHEF karyotyping and interdelta PCR-based methods can be used to differentiate between *Saccharomyces* wine yeast strains (Hoff 2012). The phylogenetic analysis of the CHEF gel electrophoresis and interdelta PCR results revealed a close genetic relatedness of the selected natural strains to the *S. cerevisiae* type strain CBS 1171 (Fig. 7a). The data indicated that the strains are distinctly different isolates with the industrial strain MH1000 clustering separately from the natural strains (Fig. 7a). CHEF karyotyping can also be used to indicate whether the progeny generated were hybrid strains or homozygous

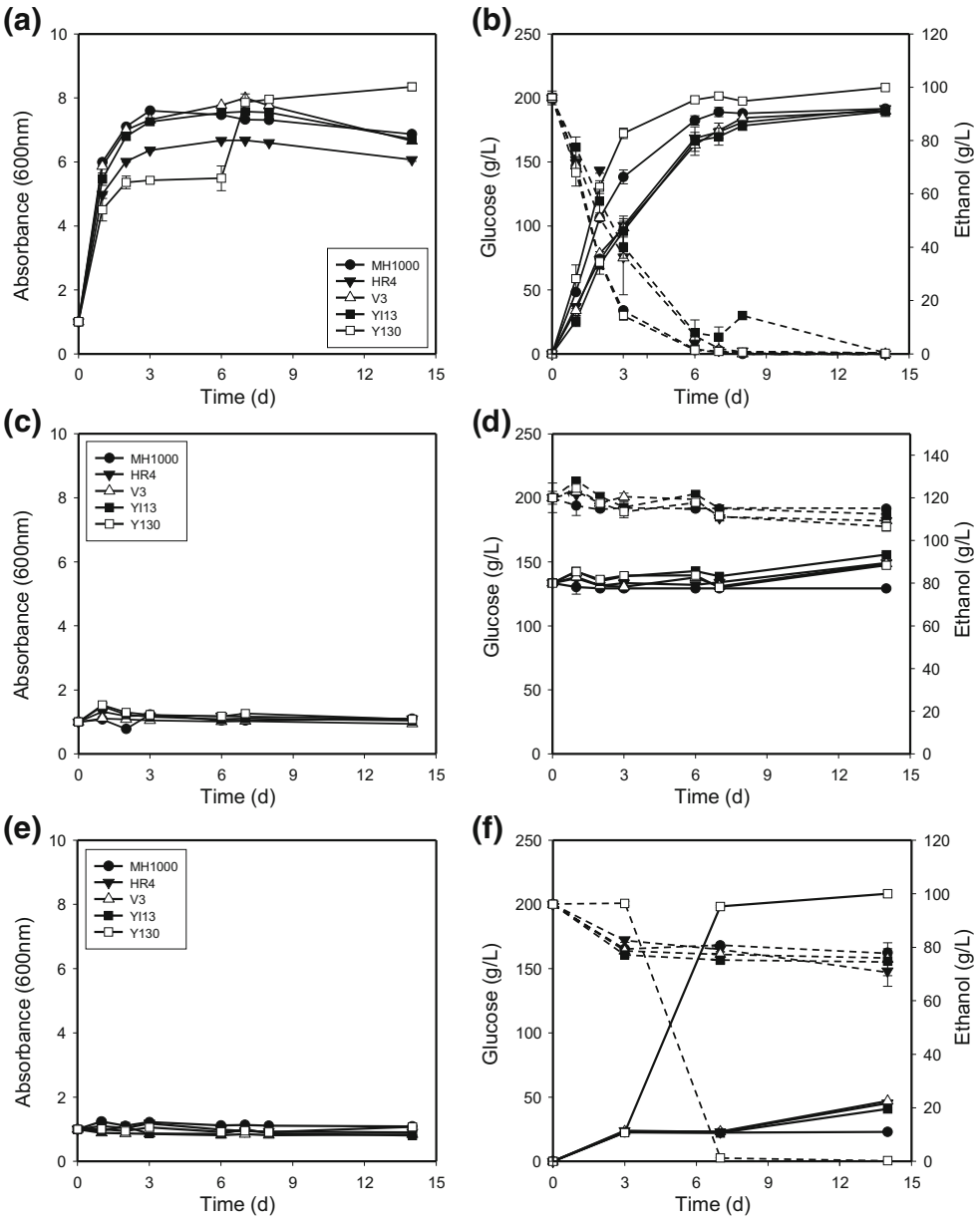

Fig. 3 Oxygen-limited fermentations of parental strains in MNS medium. Cell growth [depicted in the right side panel (**a**, **c**, and **e**)] was measured spectrophotometrically at A_{600}nm. Growth in (**a**) MNS medium, (**c**) MNS medium with 10% ethanol, and (**e**) MNS medium with 25% inhibitor cocktail is indicated. Ethanol production [depicted in the left side panel (**b**, **d**, and **f**)] was monitored by HPLC. Residual glucose is indicated by the broken line and ethanol production by the solid line. Fermentations performed in (**b**) MNS medium, (**d**) MNS medium with 10% ethanol, and (**f**) MNS medium with 25% inhibitor cocktail are indicated. Data series and error bars represent the mean values and the standard error of biological triplicates. Data points where error bars are not visible are due to low variation in biological triplicates

parental strains (Fig. 7b–f). The natural parental strains used in this study are most likely homothallic. Thus, three scenarios are possible when performing spore-to-spore mating: (1) outcrossing—hybrids are produced when two spores of opposite mating types and different genetic structures mate; (2) haplo-selfing—homozygous strains are produced when a single spore germinates, changes mating type, and mates with itself; and (3) genetically diverse strains are produced due to chromosomal or genetic rearrangement. The data indicated that the HR4/YI13 and V3/YI13 strains were all genetically diverse (Fig. 7b and c). Mating between *S. cerevisiae* strains HR4/YI30 and V3/YI30 generated four homozygous strains and one hybrid strain (HR4/YI30#4 and V3/YI30#2, respectively) for each mating (Fig. 7d and e). Homozygous strains were generated for the YI13/YI30 mating (Fig. 7f).

Discussion

The cost-effective production of second-generation bioethanol requires that the fermenting organism is osmo-, ethanol-, thermo-, and inhibitor-tolerant. Natural *S. cerevisiae* strains display a diverse range of phenotypic characteristics that could be improved through targeted breeding. In this study, natural *S. cerevisiae* strains were evaluated for phenotypic diversity, where after hybridization through spore-to-spore mating was performed in order to improve the phenotypic diversity of the isolates. Heterosis has previously been shown for *S. cerevisiae*; however, most studies focus on heterosis between laboratory and industrial strains (Marullo et al. 2009), strains that have been genetically engineered (Benjaphokee et al. 2012), or strains that have been altered via mutagenesis

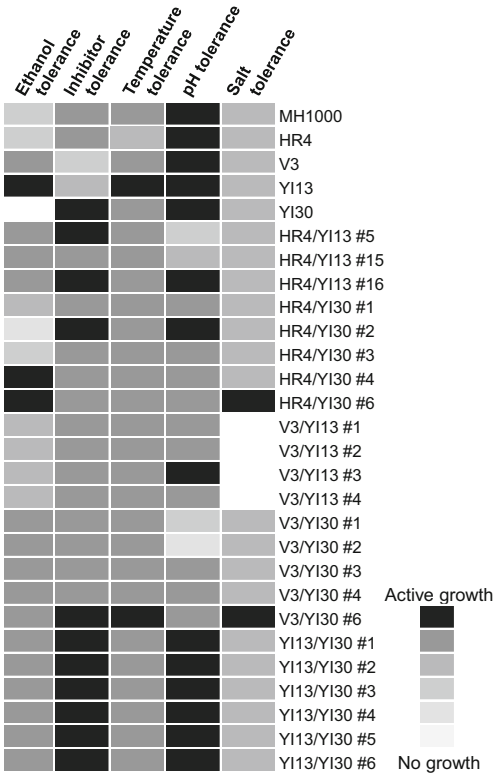

Fig. 4 Comparison of phenotypic diversity of the parental strains to the generated hybrid strains. The phenotypic diversity of the parental strains to different environments (ethanol, inhibitor, temperature, pH, and salt) was compared to that of the progeny strains. The phenotype tolerance was scored as follows: where multiple conditions for a single environment was tested (temperature, ethanol, pH, and osmolarity), a score of 6 indicates growth after 3 days at the highest dilution for all the conditions tested and a score of zero indicates no growth after 3 days for the undiluted sample for all the conditions tested. Where a single condition for an environment was tested (inhibitor), a score of 6 indicates growth within 1 day at the highest dilution and a score of zero no growth after 3 days for the undiluted sample (adapted from Kvitek et al. 2008)

(Snoek et al. 2015). This study focuses specifically on improving natural *S. cerevisiae* strains through heterosis.

Evaluation of strains obtained from different geographically located vineyards yielded strains with diverse phenotypes. High tolerance to ethanol (20% *v*/*v*), temperature (45 °C), pH (pH 2–11), and lignocellulosic inhibitors (25%) was observed in different strains. Breeding of these natural strains generated progeny displaying heterosis as well as novel phenotypes. Improved growth in various environments including low pH (pH 2) and high salt concentrations (2mol/L NaCl) was observed. Ethanol productivity was maintained, including productivity in the presence of an inhibitor cocktail.

During complete aerobic conditions and limited glucose concentrations, *S. cerevisiae* uses respiration as the preferred metabolic method with a high production of biomass and lower production of ethanol (Bakker et al. 2001). When oxygen availability is low, the yeast metabolism shifts to a respirofermentative state, with a decrease in biomass production and an increase in ethanol production (Bakker et al. 2001). During anaerobic fermentation, maximum ethanol production with low biomass yield is observed (Bakker et al. 2001). Thus, ethanol production increases as the oxygen availability decrease, with maximum ethanol production achieved at minimum oxygen availability. This explains the higher biomass ($A_{600\ nm}$ 9–15 vs 6–9; Figs. 2a and 3a) and lower ethanol yield (60–80% vs 81–89% of theoretical yield; Figs. 2b and 3b) during aerobic and limited oxygen conditions, respectively. The inability to catabolize ethanol after glucose has been depleted may be due to a decrease in the pH of the medium. The synthetic medium was not buffered so as not to influence the results obtained in subsequent fermentations where the effect of ethanol and the synthetic inhibitor cocktail was investigated.

Stress tolerance in *S. cerevisiae* is due to the ability of the strains to adjust their metabolism in order to maintain cell viability. Several mechanisms are employed including amino acid biosynthesis, transportation, energy metabolism, and membrane integrity and fluidity (Zhao and Bai, 2009). A collective response is thus needed to survive stressful conditions. The two major mechanisms whereby extreme environments/toxic compounds affect cell growth and viability are due to the effect of these conditions/compounds on cellular membranes and proteins. Harmful compounds and harsh environments affect cellular membranes by altering the permeability, thus affecting cell growth and viability. Extreme conditions have a denaturing effect on proteins compromising activity and integrity. This results in a decrease in metabolism and biosynthesis, affecting overall cell viability and cell growth (Benjaphokee et al. 2012).

Ethanol has multiple effects on yeast cells. Exposure to ethanol activates the heat shock response in *S. cerevisiae*. This leads to changes in the fatty acid composition of cell membranes, the expression of various heat shock proteins, and accumulation of trehalose (Piper 1995; Ding et al. 2009). Trehalose reduces membrane permeability and ensures proper protein folding (Ma and Liu 2010; Ding et al. 2009). The major targets of ethanol include cellular membranes and protein and enzyme activity, thus both cell growth and viability is affected as well as cellular metabolism (Ding et al. 2009; Stanley et al. 2010). Mitochondrial membranes are also disrupted leading to respiratory deficiency and thus decreased cell growth (You et al. 2003). The detrimental effect of ethanol on cell growth can be seen when comparing aerobic growth in the absence (Fig. 2a) and presence (Fig. 2b) of ethanol. There was a significant decline in cell growth (maximum $A_{600\ nm}$ of 5 compared to 15). Thus, the growth of the strains was severely compromised in the presence of ethanol. Cell stress caused by the presence of ethanol increases the demand for ATP in order to produce energy for the stress response to counteract the stress condition. Thus, more ATP is generated with a concomitant decrease in biomass production.

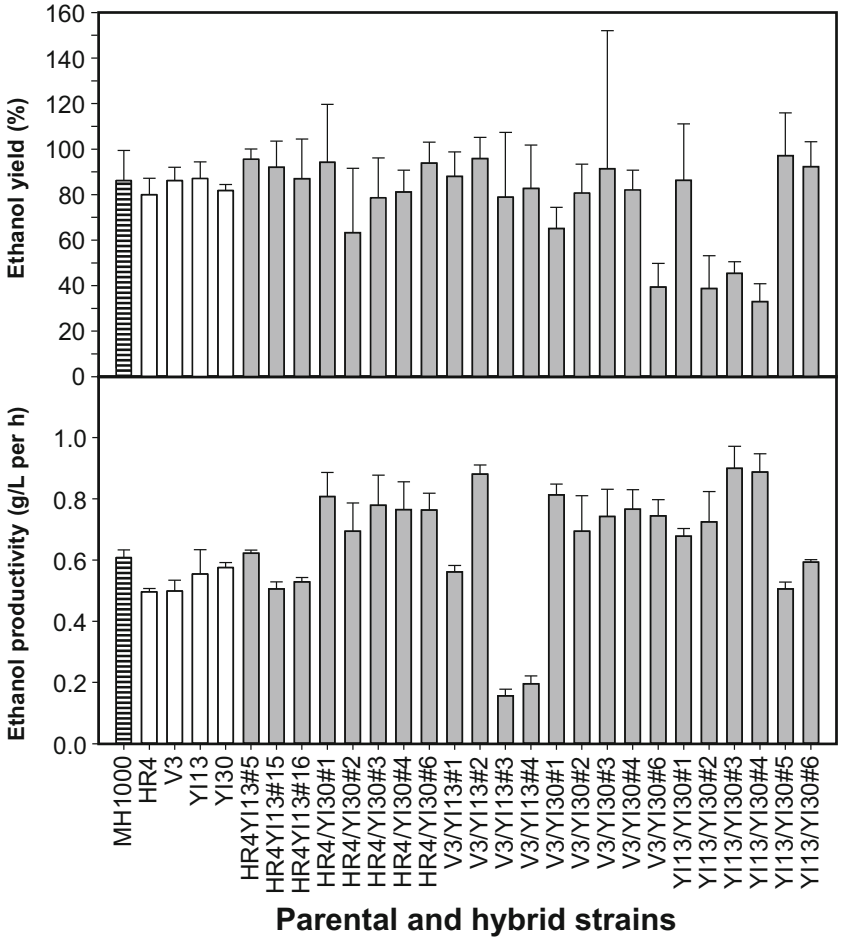

Fig. 5 Comparison of ethanol production of parental strains and generated progeny in minimal medium in a limited oxygen environment. The ethanol yield (g/L) was calculated from the slope of the curve of the fitted straight line obtained after plotting the ethanol produced (g/L) against the amount of glucose utilized (g/L). The maximum theoretical ethanol yield per gram of glucose is 0.51 g/L and was assigned as 100%. The ethanol productivity (g/L per h) was calculated as the maximum amount of ethanol (g/L) produced divided by the total time (h) taken to produce the ethanol

S. cerevisiae strains are ethanologenic and can tolerate self-produced ethanol concentrations as high as 20% (*v*/*v*) (Rose 1980). Groot et al. (1992) proposed that the maximum ethanol concentrations wherein yeast cells grow and where ethanol is produced are different and ethanol productivity could be obtained at a higher ethanol concentration than yeast cell growth. Self-produced ethanol allowed for the gradual adaptation of the strain to the high ethanol environment, thus permitting a higher accumulation of ethanol. Conversely, when the *S. cerevisiae* strains were exposed to an initial high ethanol

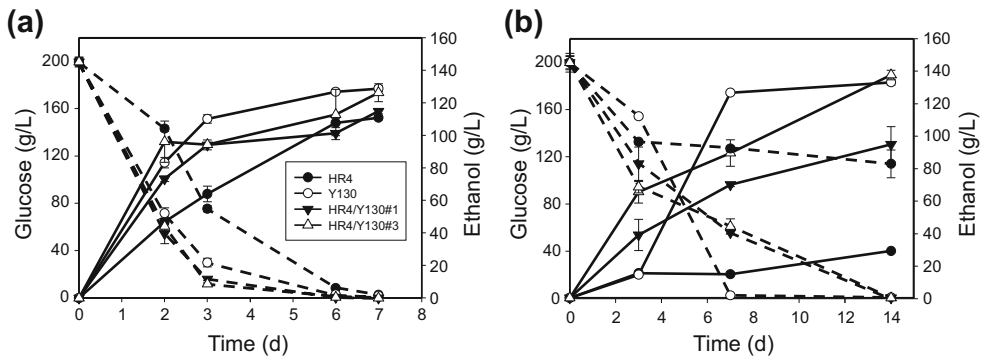

Fig. 6 Comparison of fermentation ability of parental strains and generated progeny in the presence and absence of a 25% inhibitor cocktail. Graph (**a**) represents conditions in the absence of the inhibitor cocktail and graph (**b**) represents conditions in the presence of the inhibitor cocktail. Residual glucose is indicated by the broken line and ethanol production by the solid line. Data series and error bars represent the mean values and the standard error of biological triplicates. Data points where error bars are not visible are due to low variation in biological triplicates. Data for only the inhibitor-tolerant strains are shown

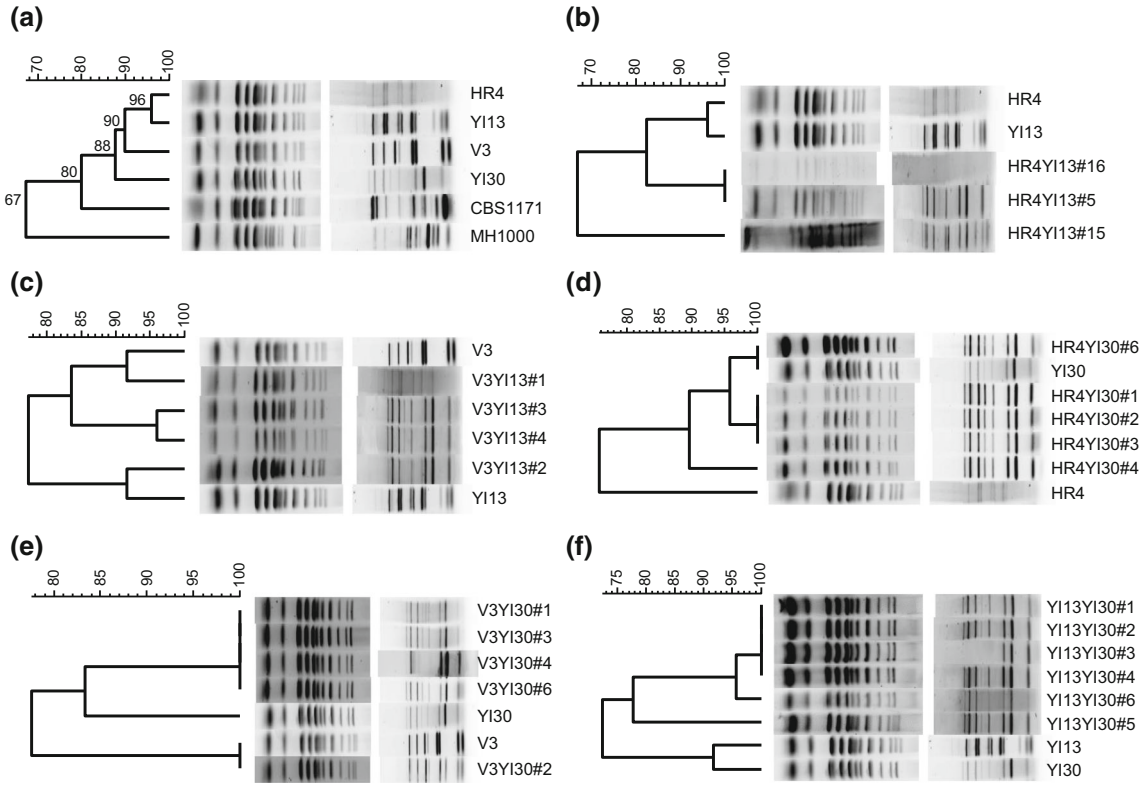

Fig. 7 Dendrogram indicating the relatedness of selected strains. Clustering of the selected strains relative to the *S. cerevisiae* reference strain (CBS 1171) and the industrial *S. cerevisiae* strain MH1000 (**a**) as well as the progeny strains relative to the parental strains (**b**–**f**) is indicated. The dendrogram was constructed by numerical analysis of CHEF karyotypes. Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA). Similarities between strains were calculated based on the Dice coefficient

concentration (10% *v*/*v*), it led to a significant loss of cell viability and hence no additional ethanol was produced as observed in Figs. 2d and 3d.

Lignocellulosic inhibitor-derived tolerance in *S. cerevisiae* is attributed to two main mechanisms, namely preventing the inhibitors from entering the cell and the detoxification of the inhibitory compounds (Liu 2011). The inhibitor tolerance mechanism depends on the inhibitors present (Almeida et al. 2007) and the environmental conditions including the availability of oxygen (Horváth et al. 2003). Furans cause a reduction in cell growth due to the inhibition of dehydrogenase activity and thus glycolysis (Pienkos and Zhang 2009). The detoxification mechanism for furfural under aerobic and anaerobic conditions differs; in aerobic conditions, furfural is converted to furoic acid; however, under anaerobic conditions, furfural is converted to furfuryl alcohol (Horváth et al. 2003). This may account for the ability of HR4 to ferment in the presence of inhibitor cocktail under aerobic conditions, whereas YI30 was able to ferment in the presence of an inhibitor cocktail in oxygen-limited conditions. Aromatic and phenolic compounds can penetrate cell membranes leading to a loss of membrane integrity. Membrane disruption affects the expression and activity of membrane transporters, which leads to a decrease in cell growth and viability (Klinke et al. 2004). Weak acids present in the inhibitor cocktail contribute to the acidification of the cytosol, increasing the demand for ATP and/or causing insufficient ATP production due to the uncoupling of the respiratory chain and the oxidative phosphorylation of ADP (Jönsson and Martín 2016). This leads to an increase in glycolytic activity in order to generate sufficient ATP, thereby decreasing biomass production (Jönsson and Martín 2016). This decrease in biomass can be seen in Figs. 2e and 3e for all of the strains grown in the presence of inhibitor cocktail whether growth occurred aerobically or in oxygen-limited conditions. The decrease in growth was more apparent when strains were grown in an oxygen-limited environment, which was expected as aerobic growth does allow for a higher biomass production. A decrease in ethanol production was expected when growing microorganisms in the presence of lignocellulosic hydrolysate. This was contrary to what was observed during this study, with two strains (YI30 and HR4/YI30#3) displaying similar ethanol production in the presence and absence of inhibitory compounds (Fig. 6). This was surprising since disruption of membrane processes including the proton-motive force and associated energy production requires changes in cellular metabolism to increase ATP production. ATP is necessary for survival and cellular repair and pumps toxins against a concentration gradient outside of the cell.

The effect of high temperatures on yeast cells is similar to ethanol exposure. Extreme temperature activates

the heat shock response with the production of heat shock proteins (Benjaphokee et al. 2012). Proteins are denatured and membrane fluidity and integrity is compromised thereby affecting metabolism and growth (Benjaphokee et al. 2012). The accumulation of trehalose attributes to temperature tolerance in yeasts possibly due to the protective effect it has on cell membranes and proteins (Neves and François 1992; Sampredo and Uribe 2004). *S. cerevisiae* is the thermo-tolerant species within the *Saccharomyces* sensu stricto group, hence growth at 45 °C for strains YI13 and V3/YI30#6 was not unexpected, as this is the maximum growth temperature observed for this organism.

Exposure to high salt concentrations causes ionic and hyperosmotic stress, which activates the high osmolarity glycerol (HOG) as well as the calcineurin pathway (Gibson et al. 2007; Matsumoto et al. 2002). The HOG pathway controls osmotic adaptation and the calcineurin pathway regulates homeostasis (Gibson et al. 2007; Matsumoto et al. 2002). Osmotolerance is also attributed to membrane stability (Mille et al. 2005). Modifying the cell membrane by altering the membrane lipid composition allows the cell to control the flux of solutes across the cell membrane (Mille et al. 2005). Growth at 1.5mol/L NaCl for several strains including heterosis with growth at 2mol/L NaCl for one hybrid strain (HR4/YI30#6) was observed in this study. This is higher than the previously reported maximum salt concentration of 1.5mol/L NaCl (Jönsson et al. 2013).

Phenotypic diversity may be due to several mechanisms including the expression of multiple genes (chromosomal duplication), interactions between the gene products (epigenetics), as well as the environment (Cubillos et al. 2011). Chromosomal duplication due to whole genome duplication or the loss/gain of individual chromosomes is selected for or induced during specific conditions (Matzke et al. 1999; Yona et al. 2012; Pavelka and Rancati 2013; Storchova 2014). This allows for structural and epigenetic alterations, thus directly influencing phenotypic variation (Matzke et al. 1999; Yona et al. 2012; Pavelka and Rancati 2013; Storchova 2014). Chromosomal duplication allows for accelerated chromosome missegregation thereby promoting chromosomal rearrangement and the development of variable karyotypes (Pavelka et al. 2010; Storchova 2014). Variations in non-coding regions allow for phenotypic variation due to differential gene expression (Zörgö et al. 2012; Salinas et al. 2012; Bergström et al. 2014; Salinas et al. 2016). Variation in regulatory regions causes changes in gene expression levels by affecting the binding affinity of transcription factors (Salinas et al., 2016). Various transcription factors have been implicated in inhibitor tolerance in *S. cerevisiae* strains (Chen et al. 2016). Gene content, copy number variations, and a third of quantitative trait loci for ecological variations are in subtelomeric regions, thus these regions are a major site for structural changes (Zörgö et al. 2012; Salinas et al. 2012; Bergström et al. 2014). Chromosomal duplication and/or epigenetics may be responsible for the phenotypic variation observed in the progeny generated as spore-to-spore mating could directly contribute to an increase in conditions leading to these phenomena.

Heterosis is the improved or increased function of cross-bred organisms in comparison to the parental organism. Outcrossing increases the possibility for allele selection thereby increasing the potential for enhanced vigor. Heterosis is due to several mechanisms including gene interactions (dominance, over-dominance, conversion of heterozygosity to homozygosity, and epistasis), variation in gene expression, protein metabolism, and metabolic efficiency (Goff 2011; Steensels et al. 2014; Segrè et al. 2005; Moore 2005; Lehner 2011). Spore-to-spore mating generates genetic diversity due to changes in ploidy (i.e., gene dosage), meiotic recombination, and chromosomal rearrangement and genetic interactions (Cubillos et al. 2011; Billiard et al. 2012). These mechanisms may lead to the generation of novel phenotypes not observed in parental strains.

It is important to note that the genetic structure of the parental strains is not known. The natural strains used in this study were able to sporulate suggesting that the strains were diploid (or polyploid). These strains may also be homothallic and since no genetic manipulations were performed, the *HO* endonuclease gene may have enabled mating-type switching. Both these scenarios indicate that the spores generated during this study could be genetically unstable. This genomic instability may have increased the possibility of meiotic recombination and chromosomal rearrangement allowing for the heterosis observed. Extensive genetic analysis of the strains is needed to elucidate the exact mechanisms responsible for the heterosis observed in the generated progeny.

Conclusion

Natural occurring organisms display diverse phenotypic characteristics that differ from conventional characteristics displayed by known isolates from a specific species. Stress tolerance and ethanol productivity of natural strains are varied and it is possible to obtain strains that are superior and more diverse than industrial strains. These strains can be exploited in industrial applications allowing for a higher productivity, diversity, and adaptability within the industrial process. The molecular mechanisms responsible for tolerance to various conditions may be due to the ability of organisms to maintain or adapt cellular membrane integrity

and fluidity as well as metabolic activity by protecting proteins against the denaturing effects of various extreme conditions.

This study indicates that unique phenotypes do exist in nature and that an artificial spore-to-spore mating method can be used to increase yeast diversity. However, the specific mechanisms that underlie these diverse phenotypic traits are still unclear. Extensive genetic characterization of the individual isolates is needed to elucidate the various mechanisms responsible for the phenotypic variations observed.

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