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MINI-REVIEW

Stress modulation as a means to improve yeasts for lignocellulose bioconversion

B. A. Brandt[1] · T. Jansen[1] · H. Volschenk[1] · J. F. Görgens[2] · W. H. Van Zyl[1] · R. Den Haan[3]

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Abstract

The second-generation (2G) fermentation environment for lignocellulose conversion presents unique challenges to the fermentative organism that do not necessarily exist in other industrial fermentations. While extreme osmotic, heat, and nutrient starvation stresses are observed in sugar- and starch-based fermentation environments, additional pre-treatment-derived inhibitor stress, potentially exacerbated by stresses such as pH and product tolerance, exist in the 2G environment. Furthermore, in a consolidated bioprocessing (CBP) context, the organism is also challenged to secrete enzymes that may themselves lead to unfolded protein response and other stresses. This review will discuss responses of the yeast *Saccharomyces cerevisiae* to 2G-specific stresses and stress modulation strategies that can be followed to improve yeasts for this application. We also explore published –omics data and discuss relevant rational engineering, reverse engineering, and adaptation strategies, with the view of identifying genes or alleles that will make positive contributions to the overall robustness of 2G industrial strains.

Keypoints

- *Stress tolerance is a key driver to successful application of yeast strains in biorefineries.*
- *A wealth of data regarding stress responses has been gained through omics studies.*
- *Integration of this knowledge could inform engineering of fit for purpose strains.*

Keywords *Saccharomyces cerevisiae* · Consolidated bioprocessing · Second-generation biofuel · Stress modulation

Introduction

All modern economies are challenged to develop a bioeconomy for sustainable future production of green bioenergy and biochemicals. Current fossil fuel and chemical production and use is linked to contaminating soil, air, and water sources, including the substantial addition of greenhouse gases to the atmosphere and consequent climate change (Correa et al. 2019). Adequate cessation of global warming is expected to require an increase in green energy usage from 2016's amount of 9.7 x 10^6 GJ d^{-1} to 46 x 10^6 GJ d^{-1} in 2040, and account for 16% of the total transport fuels (Correa et al. 2019). Plant biomass as a feedstock to produce high-density biofuels, chemicals, and other substances is thus crucial to ensure a future economy that can survive on low-carbon activity. Increased biofuel contributions to the global energy supply can further yield improved energy security, improve trade balances by limiting oil imports, and make positive contributions to rural upliftment in developing countries (Van Zyl et al. 2011; Vohra et al. 2014). Initial approaches focused on the production of biofuels from easily accessible food crops such as bioethanol produced from corn grain and biodiesel from soybeans—the so-called first generation (1G) biofuels (Hill et al. 2006). However, large-scale 1G biofuel production could potentially impact food supplies if not well managed. Additionally, the demand for liquid fuels could not be met from these sources alone.

Lignocellulosic biomass (LCB) is the most common source of renewable carbon in nature and is available in large quantities at a relatively low cost (Saini et al. 2015; Claes et al.

✉ R. Den Haan
rdenhaan@uwc.ac.za

[1] Department of Microbiology, Stellenbosch University, Stellenbosch, South Africa

[2] Department of Process Engineering, Stellenbosch University, Stellenbosch, South Africa

[3] Department of Biotechnology, University of the Western Cape, Bellville, South Africa

2020). Lignocellulose is therefore seen as a sustainable renewable feedstock for fuel production that can be utilized without affecting food production, while also having environmental benefits in comparison to petroleum- and food-based biofuels (Van Zyl et al. 2011). LCB consists mainly of cellulose, lignin, and hemicellulose that are bound in a recalcitrant structure and evolved to be resistant to degradation. Therefore, physical, biological, or chemical pre-treatment methods are required to enable the enzymatic release of simple sugars that can be converted to desired products by microbes; a process termed second-generation (2G) biofuel production.

Addition of exogenous depolymerisation enzymes incurs added expense to 2G biofuel production, rendering the overall process less cost-efficient (Saini et al. 2015). Consequently, the possibility of consolidating all biological steps in the bioethanol production process has been considered, by employing a CBP microorganism or consortium that has cellulolytic activity and ethanol-producing capabilities (Den Haan et al. 2015; Lynd et al. 2017). Challenges facing CBP include sufficiently high levels of the enzyme production without compromising ethanol productivity, co-fermentation of all available LCB sugars, and tolerating harsh fermentation environments (Den Haan et al. 2015). The industry standard fermentation yeast, *Saccharomyces cerevisiae*, remains the preferred microorganism in various biofuel production configurations (Jansen et al. 2017). While this yeast is generally considered to be more robust in industrial applications than other microorganisms, it faces additional challenges in 2G ethanol and biochemical production processes as the inherent nutrient limitations and product stresses are heightened by the presence of pre-treatment-derived microbial inhibitors. In addition, the heterologous production of enzymes to broaden the yeast's substrate range imposes a metabolic burden (Van Rensburg et al. 2012).

This review will focus on yeast responses to 2G-biofuel specific stresses and stress modulation strategies that can be followed to improve yeasts for LCB conversion processes. We also investigate published –omics data and discuss relevant rational engineering, reverse engineering, and adaptation strategies, with the view of identifying genes or alleles that will make positive contributions to the robustness of 2G industrial yeast strains.

Second-generation conversion of LCB to bioethanol

Lignocellulose is the most abundant organic biomass on Earth, with an estimated production of about 181.5 billion tonnes annually (Dahmen et al. 2019). This organic matter constitutes the major structural components of woody and non-woody plants, and its constituents have chemical properties of great biotechnological value (Howard et al. 2003). Lignocellulose is composed of a complex assembly of polymers, namely cellulose, hemicellulose, and lignin (Valenzuela-Ortega and French 2019). Cellulose is a homopolymer of β-(1,4)-glycosidic bonded D-glucose units. These long chains of glucose units are packed tightly into microfibrils that are linked to one another via hydrogen bonds to yield an insoluble crystalline cellulose structure (Zoghlami and Paës 2019). Cellulose structures are protected by coating with hemicellulose and polyphenolic lignin, where the former consists of various monosaccharide subunits, and the latter of phenylpropanoid building blocks which lends structural rigidity and hydrophobicity to the overall plant cell wall (Valenzuela-Ortega and French 2019). The accessibility of enzymes to hydrolyse the glucose units in the microfibrils are restricted by the presence of hemicellulose and lignin (Valenzuela-Ortega and French 2019; Zoghlami and Paës 2019). As such, feedstocks must undergo pre-treatment to release the fermentable sugars to be used in the fermentation process (Olson et al. 2012).

Pre-treatment of LCB is directed at destabilising the rigid plant cell wall which allows cellulolytic enzymes to gain access to the individual cellulose and hemicellulose polymers packed therein (Wang 2015; Valenzuela-Ortega and French 2019). Pre-treatment also aims at minimizing by-product inhibition that may occur during the subsequent operations in the production process (Mbaneme-Smith and Chinn 2015). Common pre-treatment processes include physical, chemical, physicochemical, and biological methods as well as combinations of these (Fatma et al. 2018), with the specific process being tailored to the type of biomass used. A pre-treatment protocol is regarded as effective, when it (i) creates simple sugars, (ii) makes enzymatic hydrolysis easier, (iii) minimizes degradation of carbohydrates, (iv) minimizes the formation of inhibitors, and (v) is cost-effective. Unfortunately, pre-treatment methods are cost-intensive, with a projected contribution of up to 40% to the overall cost of the entire bioethanol production process (Branco et al. 2019). Additionally, during pre-treatment of lignocellulosic feedstocks, inhibitory compounds are generated that negatively affect the saccharolytic enzymes and fermenting microorganisms, reducing product yields (Davison et al. 2016; Branco et al. 2019). Inhibitory compounds, such as phenolic compounds, furan aldehydes, and weak acids, are produced from lignin and hemicellulose degradation, respectively (Kim 2018). Conducting fermentations using high biomass loadings can thus greatly impair yeast cells through exposure to high osmolarity and high concentrations of toxic compounds (Caspeta et al. 2015). The presence of inhibitors thus necessitate expensive detoxification procedures. Alternatively, microorganisms can be evolved or engineered to be more robust, making the overall production process more cost-effective.

The sugars released from the cellulose and hemicellulose fractions of LCB are used for biofuel production through fermentative processes (Valenzuela-Ortega and French 2019). Thus, after the cellulose and hemicellulose fractions have been

made accessible to enzymes through pre-treatment, they are broken down to monomers through enzymatic saccharification (Saini et al. 2015). Enzymatic hydrolysis is cost-effective compared to acid or alkaline hydrolysis as it has high yields, while requiring much less equipment maintenance. The hydrolysis of cellulose is initiated by endo-β-glucanases which act on surface cellulose chains, hydrolysing random β-1,4 linkages to decrease chain length and provide free chain ends. Exo-glucanases, such as cellobiohydrolases (CBH's) act from the chain ends, proceeding down the chain to release cellobiose, which is hydrolyzed to glucose by β-glucosidases. The majority of enzyme optimization for the hydrolysis processes focuses on cellulase activities as there are few lignin-degrading enzymes of industrial standard, while hemicelluloses are mostly broken down during pre-treatment (Passoth and Sandgren 2019).

Technologies for LCB conversion are continuously being optimized to improve the overall production process (Vohra et al. 2014). The most mature methodology for biological conversion of pretreated biomass to ethanol involves separate hydrolysis and fermentation (SHF) (Fig. 1). Here, the enzyme production, substrate hydrolysis, and fermentation of hexoses and pentoses are each conducted in separate reactors, allowing maintenance of optimal operating conditions for each process (Oh and Jin 2020). However, SHF is prone to end-product inhibition and contamination due to the accumulation of free sugar. Simultaneous saccharification and fermentation (SSF) allows for the removal of end-product inhibition, as it combines saccharification and hexose fermentation in a single reactor. While reducing production costs, maintaining optimal conditions for both saccharification and fermentation is not possible since ideally the fermentation temperature should not exceed 35 °C, whereas most commercial enzyme cocktails perform best at approximately 50°C. A further advancement is the consolidation of pentose and hexose fermentation steps, known as simultaneous saccharification and co-fermentation (SSCF). However, the ideal combination of co-fermenting microorganisms capable of fermentation of all available sugars while producing high ethanol yields has not been achieved (Saini et al. 2015; Oh and Jin 2020).

The cost of exogenous enzymes presents a major challenge, leading to the exploration of CBP for single-step hydrolysis and fermentation of sugars to yield high ethanol titers (Den Haan et al. 2015; Kroukamp et al. 2017; Oh and Jin 2020). As desirable as this technology is, several challenges hamper its implementation. The use of microbial consortia was shown to be difficult, as fermentation conditions have to be kept at an optimal that favors all of the microorganisms' growth preferences (Oh and Jin 2020). The use of a single microorganism also holds challenges, such as (i) the inefficient expression of cellulolytic enzymes for sufficient feedstock hydrolysis, (ii) limited co-fermentation of both hexose and pentose sugars, and (iii) poor tolerance to harsh 2G fermentation conditions (Den Haan et al. 2015; Cripwell et al. 2019). As no natural organism(s) with such capabilities has been identified to date, several researchers have explored engineering approaches to develop CBP organisms through native or recombinant approaches (Lynd et al. 2017). The native approach focuses on engineering or enhancing ethanol production pathways in natural cellulolytic organisms, such as *Trichoderma reesei* and *Clostridium* species. The recombinant approach focuses on engineering cellulolytic enzyme production in natural ethanologenic organisms, such as *Kluyveromyces marxianus* and *S. cerevisiae* (Olson et al. 2012; Lynd et al. 2017; Valenzuela-Ortega and French 2019). Published research suggested that the recombinant approach has been more feasible, as higher ethanol yields can be obtained. While *S. cerevisiae* is arguably the most promising CBP organism in development, it needs to overcome several hurdles before being employed as an industrial cellulose CBP

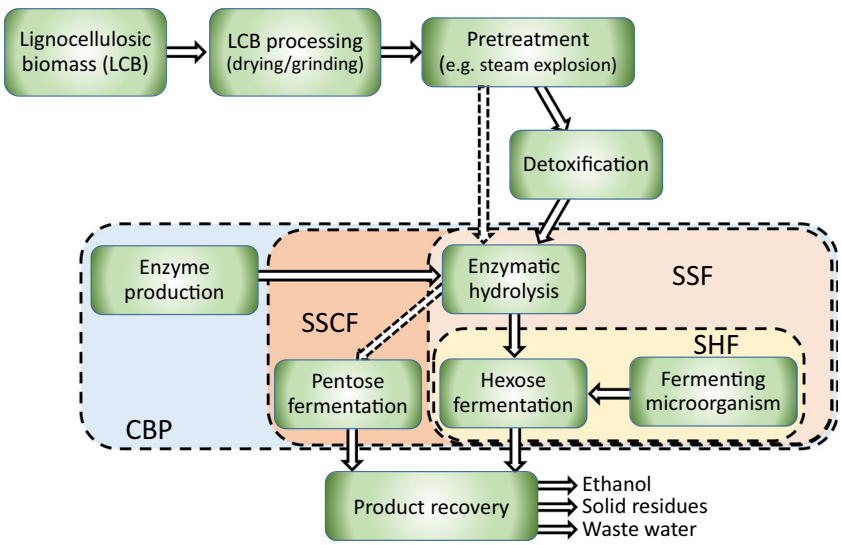

Fig. 1 Various process configurations for lignocellulose bioconversion to ethanol. Diverse processing configurations are indicated including: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF), and consolidated bioprocessing (CBP)

host. This yeast must be engineered to produce a variety of cellulases at appropriate levels, while overcoming known challenges such as hyper-glycosylation and low secretion titers. In addition, it needs to thrive in harsh fermentation conditions where osmotic, heat, and nutrient stresses will remain, in combination with additional stresses posed by toxic pre-treatment-derived inhibitors and ethanol (Wang 2015).

Overview of stresses involved in industrial fermentations

The immediate environment occupied by the yeast is constantly changing throughout the fermentation process (Walker and Basso 2020). This includes changes in the medium composition (nutrient composition and ethanol concentration) and the physical environment (temperature and pH). Yeast cells react by activating various stress responses and adapting their central metabolism (Fig. 2). This adaptation is critical to maintaining ethanol productivity and cell vitality and yeast strains that can rapidly adapt and obtain a synergism between the various stresses will thrive and proliferate in these adverse conditions. The ability of the yeast *S. cerevisiae* to quickly and efficiently adapt to a fluctuating environment is an attribute that contributes to it being the preferred host for industrial ethanol production.

The general stresses that yeast cells encounter during ethanol fermentation include osmotic and pH stress, heat stress, and nutrient starvation (Saini et al. 2018). During industrial fermentation processes yeast cells are exposed to high osmotic pressure due to high sugar concentrations, which also affect the pH of the environment. In addition, the metabolic activity of the yeast cells produces a significant amount of heat that increases the temperature of the fermentation process (Auesukaree 2017; Saini et al. 2018). Furthermore, the end-product, ethanol, is toxic to yeast cells at high concentrations (Stanley et al. 2010). Depending on the feedstock source, the nutrient content may be insufficient, specifically for micronutrients and essential minerals (de Souza et al. 2015). Moreover, the feedstock may also contain inhibitory compounds that are derived from the specific feedstock and/or the pre-treatment process (Kim 2018). Yeast can tolerate the individual stresses to a certain extent, but when combined, the overall tolerance for an individual stress factor is often reduced, leading to a decrease in cell vitality and ethanol productivity. For example, yeast cells can tolerate ethanol concentrations up to 20% ethanol v/v when optimal nutrient and

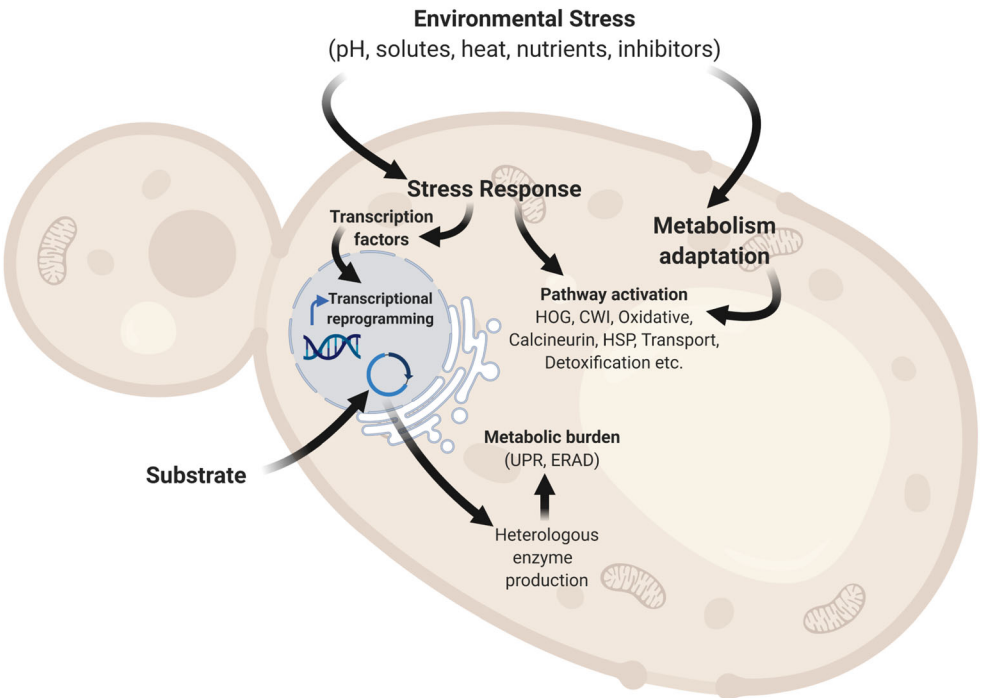

Fig. 2 A summarized schematic representation of the various stress responses associated with lignocellulosic conversion. The main processes include the activation of the stress response, as well as the adaptation of cellular metabolism to maintain cell vitality and viability. Both the stress response and metabolic adaptation lead to the activation of various pathways to maintain cellular homeostasis and thus cell viability and mitigation of cell damage. Several transcription factors are also activated that allows for transcriptional programming and thus altered gene expression to facilitate the cellular stress response. Furthermore, lignocellulosic conversion requires the introduction of heterologous genes for production of the necessary enzymes to allow for the use of various substrates. The production of these heterologous proteins may cause a metabolic burden, which leads to the activation of the UPR and ERAD. CWI – cell wall integrity pathway; ERAD – endoplasmic-reticulum-associated protein degradation pathway; HOG – high-osmolarity glycerol pathway; UPR – unfolded protein response. Schematic generated with Biorender.com

temperatures are maintained; however, the ethanol tolerance is decreased at elevated temperatures and/or poor nutrient content (Walker and Basso 2020).

Second-generation specific fermentation stresses

Various stresses are specifically relevant to lignocellulose bioprocessing such as (i) lignocellulose-derived microbial inhibitors, (ii) metabolic burden induced during cellulase production, and (iii) substrate and/or end-product inhibition. Industrial 2G fermentations are hampered by process efficiency and profitability; therefore, it is important to understand how yeast is affected by the predominant stressors typical to these constrained conditions.

Lignocellulose-derived microbial inhibitor compounds

Microbial inhibitors such as aliphatic acids, furan aldehydes, and lignin-derived phenolics result from the physicochemical degradation of lignocellulosic biomass (Kim 2018). The presence of these compounds adversely affects the fermentability of lignocelluloses by ethanologens such as *S. cerevisiae* (Taylor et al. 2012). For example, aliphatic acids such as acetic acid and formic acid can diffuse across the plasma membrane causing (i) membrane depolarization and loss of transmembrane proton gradient, (ii) cytoplasm acidification, and (iii) apoptosis (Taylor et al. 2012; Jönsson et al. 2013; Kim 2018; Hu et al. 2019). Furan aldehydes such as furfural and 5-hydroxymethyl-furfural (HMF) are derived from hexose (glucose) and pentose (xylose) sugar degradation and cause stress to yeast via the formation and accumulation of reactive aldehyde and oxygen species (ROS) (Taylor et al. 2012).

Various phenolic compounds such as *p*-coumeryl, cinnamic acid, ferulic acid, and coniferyl aldehyde are derived from the partial solubilization or de-polymerization of lignin during lignocellulose pre-treatment (Jönsson and Martín 2016; Kim 2018). These compounds exhibit toxicity based on the functional group attached to the phenolic ring (Adeboye et al. 2014; Jönsson and Martín 2016). As such, phenolic compounds have been associated with (i) increased membrane fluidity, (ii) increased cell leakage, (iii) disturbed ion and sugar transport, (iv) DNA damage, and (v) disrupting biological membrane integrity. More recently, quinone derivatives such as *p*-benzoquinone, hydroquinone, and methoxyhydroquinone have also been implicated as microbial inhibitors present in pretreated lignocellulose biomass (Cavka et al. 2015; Jönsson and Martín 2016; Yan et al. 2019). These compounds result from the oxidation of lignin-derived phenolic compounds and strongly inhibit the fermentation ability of *S. cerevisiae*. In particular, *p*-benzoquinone has been documented to completely inhibit various ethanologens including *S. cerevisiae* at concentrations between 20 and 200 mg/L (Larsson et al. 2000; Cavka et al. 2015; Yan et al. 2019). In yeast, *p*-benzoquinone can induce (i) ROS formation and accumulation and (ii) DNA damage (Yan et al. 2019).

The cytotoxic effects of microbial inhibitors can be further amplified by synergistic toxicity mechanisms, for example, the yeast cell membrane is damaged by both aliphatic acids and phenolic compounds, thus increasing cellular influx of other microbial inhibitors (Ding et al. 2011). Furthermore, multiple stressors induce ROS formation, effectively compounding its accumulation effect and resulting in increased oxidative stress and cellular damage. Due to the reactive nature of ROS (superoxide anions O_2^-, hydrogen peroxide H_2O_2, and hydroxyl radicals OH^-), it can have multiple cytotoxic effects such as (i) cytoskeletal damage, (ii) mitochondria and vacuole membrane damage, (iii) DNA damage, (iv) denaturation and damage of proteins, and (v) programmed cell death (Allen et al. 2010). As such, lignocellulose-derived microbial inhibitors represent a major technical challenge to 2G bioethanol production due to its cumulative toxicity on *S. cerevisiae* (Ding et al. 2011; Cunha et al. 2019a; Brandt et al. 2019).

Metabolic burden induced during cellulase production

Cellulose degradation to fermentable monomeric sugars requires copious amounts of cellulolytic enzymes to be produced. Furthermore, a *S. cerevisiae* strain applicable to CBP would need to produce said enzymes while maintaining fermentation in adverse conditions. The challenge, however, is that the heterologous expression of genes can exert a metabolic burden onto the host (Van Rensburg et al. 2012). The metabolic burden associated with cellulolytic enzyme expression systems has been shown to negatively affect yeast growth, by redirecting energy and cellular resources to heterologous gene expression and enzyme production (Van Rensburg et al. 2012; Ding et al. 2018). Furthermore, excessive heterologous enzyme production can induce secretion stress which triggers the unfolded protein response (UPR) and the endoplasmic reticulum-associated degradation (ERAD) mechanism (Davison et al. 2020).

The extent of metabolic burden may depend on gene dosage, gene source, secretion vs cell anchoring of heterologous cellulases, oxygen availability, and strain background (Ding et al. 2018; Davison et al. 2020). Recently, Ding et al. (2018) further elaborated on the metabolic burden experienced by *S. cerevisiae* during the overexpression of β-glucosidases from *Aspergillus aculeatus* and *Saccharomycopsis fibuligera*, by comparing stress induced by secretion versus the cell anchorage strategy. The anchorage strategy was shown to be more burdensome, leading to lower growth rate and a longer lag phase.

Substrate and/or end-product inhibition

During lignocellulosic fermentation, both the substrate (for, e.g., xylose and biomass solids) and the end-product ethanol

can induce stress in *S. cerevisiae* (Cunha et al. 2019a; Moreno et al. 2019; Osiro et al. 2019). LCB is processed through pretreatment into a solid fraction composed of insoluble solids (IS), as well as a liquid fraction containing soluble sugars such as xylose. During fermentation, the solids can negatively interact with yeast, decreasing fermentation performance by (i) deforming cellular morphology, (ii) disrupting the plasma membrane, (iii) increasing ROS formation, and (iv) altering gene expression and negating cellular stress responses (Moreno et al. 2019).

Xylose, the second most abundant sugar in 2G feedstocks, cannot be utilized by *S. cerevisiae* without introducing a heterologous xylose metabolic pathway (Cunha et al. 2019b; Osiro et al. 2019). Unfortunately, xylose metabolism under 2G fermentation conditions constrains the yeast, as the heterologous pathways are susceptible to microbial inhibitors (Deparis et al. 2017), further increasing the yeast's vulnerability (Bellissimi et al. 2009; Cunha et al. 2019a). Xylose has also been implicated in altering the expression of genes encoding gluconeogenic enzymes (Salusjärvi et al. 2006), and triggering a starvation or carbon-limited response similar to a low-glucose signal in yeast (Osiro et al. 2018; Osiro et al. 2019). This stress-derived inefficiency is detrimental to the profitability of the bioethanol production process as both glucose and xylose fermentation is required for higher ethanol titers.

Ethanol is the end product of lignocellulose bioprocessing and can also exert stress on the yeast as fermentation progresses (Deparis et al. 2017; Cunha et al. 2019a). Ethanol can alter membrane fluidity, membrane composition (Henderson and Block 2014) and reduce membrane H^+-ATPase activity. These pleiotropic effects limit cell vitality and growth (Deparis et al. 2017). A minimal ethanol titer of 4–5% v/v is required to render distillation economically viable; however, ethanol concentrations in lignocellulose fermentations struggle to reach these concentrations (Viikari et al. 2012).

Yeast general environmental stress response and other specific stress responses

The cell membrane is in direct contact with the extracellular environment and as such is the first line of defense against environmental changes (Qi et al. 2019). The lipid composition of the cell membrane is altered in response to external environment changes and protects the cell against the environmental fluctuations by adapting membrane permeability and fluidity. The various lipids and membrane-spanning sensor molecules also trigger signal transduction pathways that are responsible for the activation of various stress response pathways as well as transcriptional reprogramming, referred to as the cell wall integrity pathway (Kock et al. 2015).

Once activated, the general stress response protects the cell against several environmental stresses, including oxidative, pH, heat, and osmotic stresses and involves the induction of hundreds (~900) of genes in response to these stresses via the action of a broad range of transcription factors (Fig. 3) (Gasch 2007). Transcription factors that are essential in regulating the various stress responses required for environmental adaptation include those involved in the general stress response (MSN2/4) (Gasch et al. 2000; Vamvakas et al. 2019), oxidative stress response (YAP family of transcription factors) (Nguyên et al. 2001), nutrient stress response (GCN4) (Gasch et al. 2000; Yang et al. 2000; Natarajan et al. 2001), heat shock response (heat shock factor, HSF) (Bianchi et al. 2018), proteasome degradation pathway (RPN4) (Wang et al. 2010; Bubis et al. 2020), and drug resistance (PDR1/3) (Mamnun et al. 2002; Caspeta et al. 2015). Activation by these transcription factors leads to the accumulation of several stress proteins and metabolites that allow for adaptation to changes in the environment. The regulatory functions of these transcription factors are interdependent and along with the adaptation of the central metabolic pathways allow for the recycling of essential co-factors to maintain cell homeostasis and thus cell viability and productivity.

The heat shock response is activated to combat both ethanol and temperature stress by producing heat shock proteins (HSPs) to maintain protein function by acting as chaperones or assisting in protein folding (Guan et al. 2017; Eleutherio 2019). Alternatively, the HSPs assist in the degradation of malformed or non-functional proteins (Guan et al. 2017; Eleutherio 2019). The oxidative stress response consists of both a molecule-based (thioredoxin, glutathione, ergosterol) and an enzymatic (superoxide dismutase, catalase, cytochrome c peroxidase) response (Guan et al. 2017; Samet and Wages 2018; Eleutherio 2019). This allows for the detoxification of harmful compounds and the activation of damage control mechanisms (Gibson et al. 2007). Ion homeostasis is maintained via H^+-ATPases and the calcineurin pathway to regulate the intracellular pH of the cell, thereby maintaining cell viability (Matsumoto et al. 2002; Cyert 2003; Guan et al. 2017; Eleutherio 2019). The osmotic stress response is regulated by the high osmolarity glycerol (HOG) pathway which allows for osmotic adaptation via glycerol-3-phosphate dehydrogenase (GPD1) (Krantz et al. 2009; Warringer et al. 2010; Guan et al. 2017). GPD1 catalysis a crucial step in the biosynthesis of glycerol, the main osmolyte in yeast cells. The osmotic- and ion stress response pathways also affect the permeability of cell membranes; therefore, alterations in the membrane composition are induced to improve the general stress tolerance of the yeast cell. Furthermore, the accumulation of ethanol affects the fluidity of the cell membrane which is mitigated by changing the lipid composition of the cell membrane (Guan et al. 2017; Eleutherio 2019; Yin et al. 2020). The production of trehalose, an important protective

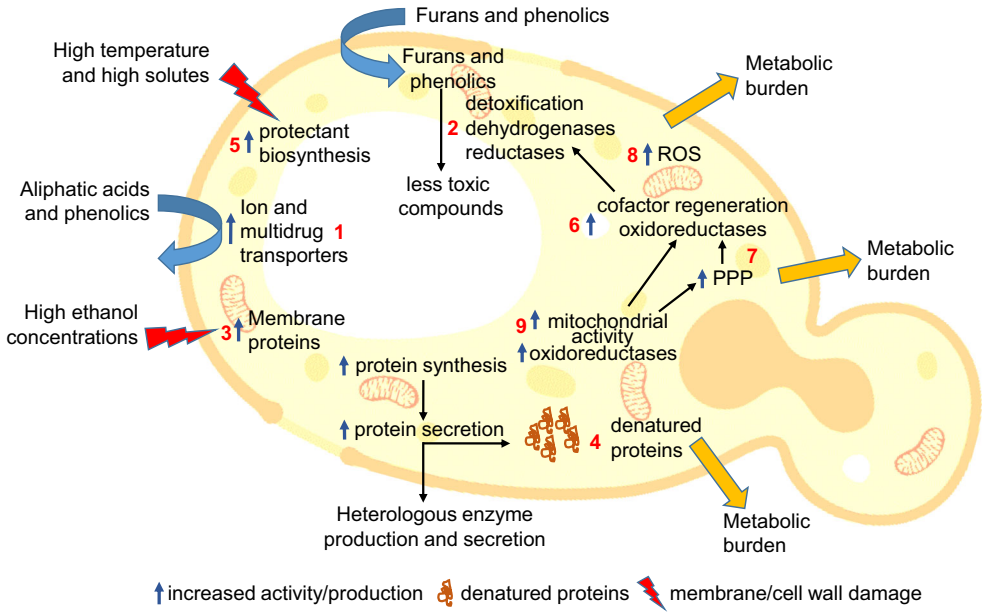

Fig. 3 A schematic representation of the second-generation fermentation stresses and the yeast's general response to these stresses. The direct mechanisms used include (1) the removal of inhibitory compounds using multidrug transporters and (2) enzymatic detoxification of inhibitory compounds. Indirect mechanisms required to combat the damage caused by exposure to inhibitory compounds, high temperature, and ethanol include (3) membrane and (4) protein damage, which requires the production of (5) protective metabolites. Other mechanisms include (6) the regeneration of cofactors to aid in the detoxification including cofactor regeneration via (7) the pentose phosphate pathway (PPP), as well as cofactor regeneration for combating (8) reactive oxygen species (ROS) accumulation due to inhibitor exposure. (Figure adapted from Brandt et al., 2019)

metabolite, is also upregulated to maintain plasma membrane integrity. Genes involved in trehalose biosynthesis (*TPS1* and *TPS2*) have therefore been implicated in stress tolerance (Guan et al. 2017; Eleutherio 2019).

It is difficult to determine which genes are part of the general stress response and which genes are related to a specific stress condition, due to cross-talk between the general stress response, central regulators of the nutritional response, and coordinators of central metabolic activities. As a consequence, both the general and stress-specific responses determine the level of overall stress tolerance. With a wealth of "-omics" information based on yeast stress responses available, the remainder of this review will explore the stress responses of yeast at a molecular level and how this information may be used to improve yeasts for various configurations of second-generation ethanol production.

Utilising omics data to unravel 2G stress responses in *S. cerevisiae*

S. cerevisiae promptly senses perturbations of its external environment to recruit a multistage cascade of processes to capacitate a unified tolerance response which buffer against a variety of stress onslaughts (Gibson et al. 2007; Deparis et al. 2017; Peltier et al. 2019). The current understanding of the subtle modulations of the stress responses in yeast has progressed from the early pioneering one-gene-one-phenotype paradigm to interconnected models of entire metabolic pathways and systems. Although the former provided fundamental insights into monogenic and simple multi-gene traits, it failed to explain the dynamics of multigene interactions of polygenic traits on a genomic scale (Deparis et al. 2017; Geng et al. 2017).

Quantitative genetic studies of 2G stress responses in *S. cerevisiae*

With advances in molecular biology, high throughput omics techniques and algorithmic agility, quantitative genetic studies such as bulk-segregant analysis of experimental genetic crosses and subsequent quantitative trait locus (QTL) analysis are powerful in concurrently identifying and filtering the genetic variations contributing to a trait. QTL analysis has been instrumental in identifying sequence variants involved in a single stressor tolerance, including ethanol-, acetic acid-, and thermotolerance (Hu et al. 2007; Ehrenreich et al. 2010; Parts et al. 2011; Swinnen et al. 2012; Cubillos et al. 2013; Pais et al. 2013; Yang et al. 2013; Duitama et al. 2014; Greetham et al. 2014, Wohlbach et al. 2014; Geng et al. 2016; Meijnen et al. 2016; Maurer et al. 2017; Fernández-Nino et al. 2018; Stojiljkovic et al. 2020; Pais et al. 2013). However, within the lignocellulose conversion environment, the yeast has to cope with the compounded effect of synergistic toxicity of complex mixtures of inhibitors simultaneously (Liu et al. 2004;

Almario et al. 2013). Recently, QTL and Genome-Wide Association Studies (GWAS) were employed to decipher the genetic determinants of multi-stressor tolerance in *S. cerevisiae* (Sardi et al. 2018; De Witt et al. 2019a).

As powerful as QTL analysis is in dissecting the genetic signatures of polygenic traits, understanding the interaction between these collections of molecular elements involved in a phenotypic trait, i.e., quantifying their individual contributions in a spatial, temporal, and regulatory setting, while unmasking epistatic and pleiotropic effects remain daunting (Ehrenreich et al. 2010; Cubillos et al. 2011; Meijnen et al. 2016; Sardi and Gasch, 2018; Sardi et al., 2018). Furthermore, although QTL analysis is a powerful technique to accurately and reproducibly predict the impact or effect of non-synonymous mutations in coding regions, the inference on the effect of alterations in regulatory (promoter and terminator) regions on expression levels is more complex. These factors have significant implications for strain engineering.

QTL analysis has led to the identification of specific known and novel causative alleles, which can be strongly genetic background dependent, or have no previously reported direct link with a specific stress tolerance. Some general themes have emerged amongst tolerance mechanisms between inhibitors spanning different classes. *S. cerevisiae* employs interconnected and coordinated tolerance mechanisms including detoxification, energy management, redox homeostasis, oxidative stress management, and DNA damage repair.

Quantitative genetic studies highlight the variability in genetic architectures underpinning complex traits between different *S. cerevisiae* genetic backgrounds (Peter et al. 2018). The current understanding of the *S. cerevisiae* tolerance system is mostly based on observations in genetically "restricted" domesticated strains, i.e., laboratory and industrial strains (Peter et al. 2018; Sardi and Gasch 2018). This realization, combined with the notion that natural isolates innately harbor greater genetic variation, has revived the interest in "wild" *S. cerevisiae* isolates. As such, the genetics of natural isolates has great potential to provide a more holistic understanding of how yeast responds to stress given that evolution and selective pressure has been tailoring genomes (Slate 2005; Peter et al. 2018). Two questions arise from the work to date; (i) when will we reach the point of having a complete holistic understanding of the complexity and magnitude of genetic interactions involved in lignocellulose inhibitor tolerance and (ii) will it still be feasible to reverse engineer tolerance traits in *S. cerevisiae*? The first question can be addressed by increasing the availability of genome sequences and phenotypic data of *S. cerevisiae* strains to include in future quantitative genetics analysis. Combined with expanded sequence space, innovative bioinformatic tools and algorithms to model and predict the genetic underpinning of complex traits, such as inhibitor tolerance, is essential (Ho et al. 2018). With regard to the second question, advances in high-throughput synthetic biology approaches such as CRISPR/Cas9 technologies and de novo synthetic genomes promise the means to genetically engineer *S. cerevisiae* at scale (Maurer et al. 2017).

Genomic alterations, as detected by QTL analyses, present only one of several layers of regulation within the cell. A mutation within a gene coding sequence or regulatory element may or may not affect the overall phenotype of the cell due to redundancy and regulatory systems. In this regard, a full view of the genome, transcriptome, and proteome is required to elucidate the mechanisms involved in a complex trait (Bai et al. 2015; Ho et al. 2018; Buccitelli and Selbach 2020).

Transcriptomic analysis to identify key stress-mediating pathways

Transcriptomic analyses has been used to elucidate the complexity of gene expression regulation in *S. cerevisiae* as it provides access to the entire range of RNA transcripts, including coding and non-coding RNAs (Manzoni et al. 2018). Analyzing the full complement of RNA transcripts provides insight into the presence or absence and quantity of a transcript, alternative/differential splicing, and sequence variations including SNPs and quantitative assessment of genotype influence on gene expression. This information is essential to understand the dynamics of cellular processes involved in metabolism (Manzoni et al. 2018).

Several transcriptomic studies performed on laboratory, industrial and genetically engineered *S. cerevisiae* strains, revealed integrated approaches for cell survival during lignocellulosic fermentations. These studies were able to identify several pathways involved in maintaining cell vitality as well as identify key role players within these pathways (Fig. 3). These include (i) pathways involved in general cell metabolism (carbon, fatty acid, and amino acid metabolism) and energy generation (Li and Yuan 2010; Liu 2011; Zhao et al. 2015; Kasavi et al. 2016; Thompson et al. 2016; Haclsalihoglu et al. 2019), (ii) pathways associated with maintaining cell wall integrity and cellular membranes (Zhao and Bai 2009; de Lucena et al. 2015; Thompson et al. 2016), (iii) pathways associated with the mitochondria (Li and Yuan 2010; Thompson et al. 2016), (iv) pathways involved in transport (Ma and Liu 2010; Kasavi et al. 2016), (v) pathways involved in stress responses including DNA damage (Zhu and Xiao 2004; Thompson et al. 2016) and oxidative stress (de Lucena et al. 2015; Zhao et al. 2015; Kasavi et al. 2016; Haclsalihoglu et al. 2019), (vi) signaling pathways such as the MAPK and PKA signaling pathways (Zhou et al. 2014; Zhao et al. 2015; Thompson et al. 2016), and (vii) pathways involved in the detoxification and removal of inhibitory compounds (Li and Yuan 2010; Ma and Liu 2010; Liu 2011; Thompson et al. 2016; Haclsalihoglu et al. 2019). These pathways allow the cell to maintain cell vitality through energy generation (i, iii), combat cellular damage caused by inhibitory compounds (ii; v, vi), and assist in stress

tolerance by removing (iv, vii) or detoxifying (pathways vii) inhibitory compounds.

In general, the various transcriptomic datasets indicated the contribution of similar pathways to specific stress indicators. However, the degree of transcriptional variation within these pathways is strain-dependent and indicative of each strain's specific innate phenotype. It is important to note that although transcriptomic data has vastly improved our understanding of the various pathways involved in any specific scenario, it is merely an indicator of the genes implicated and the results need to be experimentally verified to definitively prove the impact of any specific gene.

Proteomics of stress tolerance phenotypes

Proteomics has been established as a high-throughput and highly sensitive method to investigate the protein level changes in a global context. However, relatively few label-free MS-based proteomics studies have focused on the elucidation of the underlying mechanisms of tolerance phenotypes in *S. cerevisiae* (Salvadó et al. 2008; Lin et al. 2009; Vogel et al. 2011; Walter and Ron 2011; Nagaraj et al. 2012; Lv et al. 2014; Shui et al. 2015; Choudhary et al. 2019; Liu et al. 2019). The majority of studies employ 2D-PAGE followed by mass spectrometry or focus more on a specific methodology to increase sensitivity (Hebert et al., 2014). Furthermore, there remains minimal overlap between studies on stress tolerance, making comparisons and conclusions difficult.

Heat shock stress was investigated by Nagaraj et al. (2012) and 234 proteins known to be involved in heat shock response, accumulated to significantly altered levels. Levels of proteins involved in ribosomal biogenesis and translation also decreased under thermal stress. Time-dependent proteome analysis by Vogel et al. (2011) was correlated with transcriptomic data during oxidative stress conditions to investigate translation and protein degradation. They were able to show that the response was broadly as expected of oxidative stress; however, for a third of the proteins detected, there were large deviations between mRNA and protein level (Longo et al. 2015). Proteomics have revealed that underlying tolerance mitigation mechanisms of *S. cerevisiae* to general lignocellulose fermentation-associated stresses include (i) attenuation of pH, redox, and energy homeostasis; (ii) upregulation of stress-response proteins; (iii) lipid and cell wall biogenesis; (iv) filamentous/pseudohyphal growth and meiosis; (v) aromatic amino acid biosynthesis; and (vi) stress-protective molecules for oxidative stress mitigation (Xiao et al. 2018; Unrean et al., 2018; Li et al. 2019).

Proteomics was instrumental in the elucidation of the stress response elicited by complex lignocellulosic inhibitor mixtures and continues to play a crucial role in refining current stress response models (Ding et al. 2012; Lv et al. 2014; Unrean et al. 2018; De Witt et al. 2019b). Lv et al. (2014) were able to show that tolerance in *S. cerevisiae* to multiple inhibitors was due to an increase of several alcohol dehydrogenases and pentose phosphate pathway enzymes, which suggested that *S. cerevisiae* was able to regulate redox potential under lignocellulosic inhibitor stress conditions better. Measurements of cellular ATP and NAD(P)H indicated that the cells were able to regulate redox potential under lignocellulosic inhibitor stress conditions better. They suggested a partitioning of resources into the regulation of protein synthesis and cell growth to be one of the primary tolerance mechanisms.

In our work on lignocellulosic inhibitor tolerance in natural strains of *S. cerevisiae*, we proposed a genetic background independent proteome core response as well as a genetic background specific proteome response (De Witt et al. 2019b). We postulate that a core response is the minimum requirement for a yeast strain, regardless of genetic background, to enact a tolerance response which will provide it with intermediate tolerance characteristics to lignocellulosic inhibitor exposure. The core response included proteins well known to be involved in tolerance to multiple stresses, especially redox balancing mechanisms as the primary processes involved in lignocellulosic inhibitor tolerance. Detoxification of ROS and improved energy and redox management was also required for superior tolerance. It was shown that the background-specific response regulated proteins unique to each natural isolate. However, the unique response proteins regulated similar functional processes as the core response in both isolates, and thus, the proteome adapted by regulating the same global response processes, but with different proteins. It supports the view that a trait is regulated by any of a set of gene clusters which result in a similar response. Superior tolerance to lignocellulosic inhibitors, as observed from the core and unique protein response, is, in our view, due to the fine-tuning of core functions via strain-specific proteins.

Strategies to improve 2G yeasts: rational design approaches

Rational engineering of yeast is a powerful technique towards generating desirable phenotypes. However, the genetic determinants of the phenotype must be known, highlighting the need for omics data. One possible strategy for utilizing omics data is to identify where key genes and stress response pathways overlap in response to specific stressors, in order to engineer yeasts in such a way as to gain broader robustness to the wide spectrum of 2G stressors. Overcoming lignocellulose-derived microbial inhibitors in 2G fermentation processes has been the subject of many studies, specifically how *S. cerevisiae* can be rationally engineered to have more robust physiology and exhibit enhanced in situ detoxification of 2G pretreated materials and hydrolysates (Almeida et al. 2009; Wierckx et al. 2011; Adeboye et al. 2015; Brandt et al. 2019).

The overexpression of genes encoding enzymes with aldehyde reductase activity, e.g., *ARI1*, *ADH6*, and *ADH7*, have been well documented to confer resistance to furan aldehydes (Petersson et al. 2006; Heer et al. 2009; Jordan et al. 2011; Sehnem et al. 2013). Phenolic resistance has been linked to overexpression of genes such as *PAD1*, *FDC1*, *ATF1*, *ATF2*, and *ALD5* (Mukai et al. 2010; Lin et al. 2015; Richard et al. 2015; Adeboye et al. 2017), whereas aliphatic acid resistance has been linked to overexpression of *RCK1*, *TRX1*, and *FDH1* amongst other examples as seen in Table 1 (Hasunuma et al. 2011; Unrean et al. 2018; Oh et al. 2019). More recently, the emphasis has shifted from single inhibitor group tolerance towards mechanisms that engineer multi-inhibitor tolerance phenotypes, as this is a more realistic representation of the stresses present in the 2G bioethanol production process. Resistance to not only aliphatic acids but also corn stover hydrolysate was reported with the overexpression of *ADE1*, *ADE13*, and *ADE17* involved in de novo purine biosynthesis in *S. cerevisiae* (Zhang et al. 2019a). Similarly, enhanced multi-inhibitor tolerance phenotypes where engineered by the overexpression of *TAL1*, *ARI1*, *ADH6*, *FDH1*, *PAD1*, and *ICT1* genes in an industrial *S. cerevisiae* strain (Brandt 2019).

Rational engineering interventions to improve heterologous cellulase secretion through a variety of strategies, including stress modulation has been the topic of several studies (Kroukamp et al. 2017; Kroukamp et al. 2018). Recently, Lamour et al. (2019)

Table 1 Recent engineering strategies toward mitigating second-generation relevant stresses

Approach	Strategy	Phenotypic responses	References
Inhibitor tolerance			
Rational engineering	Overexpression of *RCK1* for acetic acid resistance	2-fold increase in specific ethanol productivity. Decreased ROS (40%)	(Oh et al. 2019)
	Co-expression on *TAL1* and *ADH1* for improved furfural resistance in xylose capable strain	Improved furfural resistance and improved ethanol production	(Hasunuma et al. 2014)
	Overexpression of *PAD1*, *ATF1*, *ATF2* and *ALD5* for phenolic resistance	Improved resistance to ferulic acid, coniferyl aldehyde, and p-coumaric acid.	(Adeboye et al. 2017)
	Overexpression of *ADE1*, *ADE13*, and *ADE17* involved in de novo purine biosynthesis.	Improved ethanol production and resistance phenotypes in synthetic and corn stover hydrolysate	(Zhang et al. 2019a)
	Deletion of *ATG22* to decrease acetic acid induced programmed cell death	Decreased amino acid starvation, ROS and intracellular acidification, improved cell wall integrity, plasma membrane permeability and fluidity. Increased transcription of HSP genes	(Hu et al. 2019)
Comparative transcipt-omics	Identify genes key to multi-inhibitor tolerance phenotypes in evolved robust strains.	Evolved strains exhibited improved cellular integrity and more robust mitochondria: identified 52 genes with possible involvement in multi-inhibitor stress response.	(Thompson et al. 2016)
Chemogenomic screening	Identify genes and pathways for phenolic resistance	Identified *ZWF1* as key gene in coniferyl aldehyde detoxification for phenolic resistance	(Fletcher et al. 2019)
Alleviating metabolic burden and secretion stress			
Rational engineering	Overexpression of *YHB1* and *SET5*	Improved heterologous secretion and environmental stress tolerance	(Lamour et al. 2019)
End-product/substrate inhibition			
Rational engineering	Heterologous expression of *pprI* gene (*Deinococcus radiodurans*)	Improved salt and ethanol tolerance	(Helalat et al. 2019)
	Overexpression of *TRK1* and *PMA1*, plasma membrane potassium and proton pumps.	Improved ethanol production and ethanol tolerance	(Lam et al. 2014)
Holistic approaches toward multi/cross tolerance			
Rational engineering	Deletion of *ADY2*, an acetate transporter gene	Improved resistance to ethanol, hydrogen peroxide and acetic acid. Improved ethanol production, decreased ROS and plasma membrane permeability.	(Zhang et al. 2017)
CRISPR	Genome shuffling using CRISPR-Cas9 to develop robust thermo-tolerant strain	Improved tolerance to ethanol, low pH and high temperature (39°C).	(Mitsui et al. 2019)

described the over-expression of *YHB1* and *SET5*, two native *S. cerevisiae* genes linked to yeast stress tolerance, and demonstrated that improved heterologous secretion and environmental stress tolerance could be engineered into yeast simultaneously. Transformants showed increased secreted heterologous CBH activity that ranged from 22 to 55% higher compared to the parental strains which did not lead to deleterious growth effects. These strains also demonstrated improved tolerance to osmotic and heat stress with lower induction of the unfolded protein response, suggesting mechanisms for enhancing enzyme production through stress modulation. Overexpression of *YHB1*, known to play roles in oxidative and nitrosative stress responses (Lewinska et al. 2008), in an industrial strain also enhanced heat and ethanol stress tolerance and improved ethanol productivity in medium containing 5 g/L acetic acid (Lamour et al. 2019).

Efficient enzyme production with the use of *S. cerevisiae* as whole-cell biocatalysts is essential for consolidated bioprocessing of 2G feedstocks. Cunha et al. (2020) illustrated the rational engineering of cellulolytic (β-glucosidase 1, *A. aculeatus*) and hemicellulolytic (β-xylosidase, *Aspergillus oryzae*; endoxylanase II, *T. reesei*) enzymes into robust industrial strains namely, Ethanol Red, PE-2, CAT1, and CA11. The yeast strains were also rationally engineered for xylose utilization, by the heterologous introduction of both the fungal reductase/dehydrogenase and bacterial xylose isomerase pathways. Remarkably, the CBP process was shown to be more effective than traditional SSF fermentation process with commercial enzyme supplementation, illustrating the potential of a robust hemi/-cellulolytic and xylose assimilating industrial *S. cerevisiae* strain (Cunha et al. 2020). As a further illustration of this point, the rational engineering of multiple *BGL3* genes of *Phanaerochaete chrysosporium* into *S. cerevisiae* M2n via δ-integration did not result in detectable metabolic burden. Metabolomics analysis revealed shifts in metabolism in this strain allowing for metabolic homeostasis, even under 2G stress conditions (Favaro et al. 2019). This highlights the importance of utilising robust yeast strains as 2G bioethanol chassis organisms.

Rational engineering has also been used in the further development of *S. cerevisiae* strains to exhibit robust phenotypes such as thermotolerance and ethanol tolerance (Gao et al. 2017; Helalat et al. 2019; Mitsui et al. 2019). The complexity of these phenotypes, coupled with the interaction of various 2G stresses on the yeast cellular viability and fermentation ability, complicates the introduction of multi/cross-tolerance phenotypes via rational engineering alone. Furthermore, yeast stress responses vary depending on the carbon source utilized (Li et al. 2020).

Strategies to improve 2G yeasts: reverse engineering and strain adaptation

A long period of natural evolution and subsequent artificial selection has led to significant genetic diversity among *S. cerevisiae* strains, resulting in stress tolerance variability. Tolerance to environmental stress is a complex phenotype influenced by the interaction of multiple gene products, several of which are not well characterized (Zhang et al. 2019b). The improvement of yeast tolerance to single or multiple stresses by rational engineering is thus challenging. Alternatively, random mutational approaches and evolutionary engineering or adaptive evolution have been implemented to select *S. cerevisiae* strains with improved stress tolerance and fermentation performance (Caspeta et al. 2015; Zhang et al. 2019b; Davison et al. 2020). These methods can be employed to obtain strains with desired phenotypes deriving from changes in multiple genes. During adaptive laboratory evolution (ALE), a microorganism is cultured under defined conditions for prolonged periods with serial dilution in fresh media to allow the selection of improved phenotypes (Dragosits and Mattanovich 2013). As the method relies on recurrent cycles of mutagenesis, recombination, and selection, molecular evolution of complex traits relying on the interaction of many genes can occur. Increases in fitness can be gauged during the evolution by screening for an improved phenotype. This technology has been applied to improve tolerance toward heat, osmotic stress, ethanol, and the microbial inhibitors generated during lignocellulose pre-treatment (Caspeta et al. 2015; Zhang et al. 2019b).

The industrial yeast strain, Ethanol Red®, underwent long-term ALE and yielded a strain able to produce ethanol from non-detoxified spruce hydrolysates (Wallace-Salinas and Gorwa-Grauslund 2013). Similarly, the technique was applied to select strains tolerant to hydrolysates containing acetate, furfural, and HMF (Liu et al. 2005; Keating et al. 2006; Heer and Sauer 2008). Strains adapted to grow in a 3 mM furfural had a reduced lag-phase and higher reductase activities for furfural conversion, indicating that faster consumption of these inhibitors was the main mechanism of improved tolerance in these strains. Microarray studies showed that the redox balance and energy state of the cell are major drivers to furfural and HMF tolerance with the *ADH6* alcohol dehydrogenase identified as a major contributor for tolerance to HMF in aerobic and anaerobic conditions (Petersson et al. 2006; Ask et al. 2013). Tolerance to furfural could be increased by overexpression of *ADH7*, *YKL071W*, and *ARI1*, all encoding reductases (Heer et al. 2009; Sehnem et al. 2013).

To improve thermotolerance, *S. cerevisiae* CENPK113-7D was evolved at high temperature to generate strains able to grow at 40°C (Caspeta et al. 2015; Zhang et al. 2019b). Thermotolerant *S. cerevisiae* strains evolved over 450 generations at 39°C had a duplication of chromosome III and overexpressed genes related to that chromosome (Yona et al. 2012). Similarly, following ALE experiments at 39.5°C, a strain was isolated with a partial duplication of Chr III containing the *HCM1* gene (Caspeta et al. 2014a). As the chromosomal duplication was eventually lost, these duplications

appear to be temporal solutions to stress (Yona et al. 2012). Chromosomal duplications were also observed in diploid strains adapted for tolerance to high pH where Chr V was duplicated. Remarkably, haploid *S. cerevisiae* populations displayed segmental duplications only. Several evolutionary engineering studies linked chromosomal copy number variation to industrially important traits, including tolerance to products or inhibitors, ethanol production, nitrogen uptake, and improved kinetics of sugar fermentation (Davison et al. 2020).

Complex stress selection is likely more suitable for the isolation of strains with enhanced fermentation performance, as the fermenting organism in 2G bioethanol production will likely face several challenges simultaneously. ALE has thus been applied to generate strains using tolerance to multiple stressors. ALE of Ethanol Red® in spruce hydrolysate at 39°C yielded strains capable of converting hydrolysates to ethanol efficiently at high temperature (Wallace-Salinas and Gorwa-Grauslund 2013). Unlike the resistance phenotype evolved with the challenge by inhibitors alone, the superior phenotype of these strains relied on the higher thermotolerance. Thermally evolved strains with improved ethanol tolerance did not overexpress *PMA1* encoding the plasma membrane H^+-ATPase; however, levels of its negative regulator Hsp30p increased upon thermal stress (Piper et al. 1997; Meena et al. 2011; Caspeta et al. 2014b). This would suggest that thermal evolution optimized ATP use for proton export, decreasing energy for maintenance. Electrical potential and proton fluxes could then decrease the energy of ATP hydrolysis for proton excretion to enhance resistance to ethanol (Caspeta et al. 2015). Strains evolved via different stresses often display similar adaptation mechanisms when analyzed at the genomic or metabolomic level. The correlations among mutant strains evolved at pH 2.5, 30% glucose, or 0.8 M NaCl treatment implied that the damage or the repair mechanism associated with tolerance to stress inherent to low pH was also associated with osmotic stress tolerance (Caspeta et al. 2015).

Random mutagenesis and genome-shuffling have also been applied to improve stress tolerance, often in combination with ALE. Genome shuffling was used to increase the tolerance of a strain to heat, acetic acid, and furfural stresses, yielding a strain tolerant to 0.55% (v/v) acetic acid as well as 0.3% (v/v) furfural at 40°C (Lu et al. 2012). Genome-shuffling was also used to generate both thermal and ethanol tolerance in an industrial yeast isolate, SM-3. This strain could be used to ferment substrates with 20% (w/v) glucose at 45°C while resisting 9.5% (w/v) ethanol (Shi et al. 2009). *S. cerevisiae* strains were recently evolved for osmotic and ethanol tolerance, applicable to very high gravity fermentations (Zhang et al. 2019b). These strains were isolated after ten rounds of freeze-thaw treatment and plate screening under high osmotic and ethanol stress. The evolved strain, YF10-5, displayed a 16% higher ethanol yield than the parent strain during 35% (w/v) glucose fermentations. This strain displayed upregulation of genes encoding heat-shock proteins (*HSP26*, *HSP30*, and *HSP104*), trehalose synthesis (*TPS1*), and ethanol metabolism (*ADH1*, *HXK1*, and *PFK1*), indicating increased stress tolerance and fermentative capacity (Zhang et al. 2019b).

A combination of chemical mutagenesis and ALE over 486 generations in continuous cultivation with increasing ethanol stress was used to generate strains with improved growth rates and shorter lag phases (Stanley et al. 2010). These strains continued to flourish in ethanol concentrations that were lethal to the parental strain and showed higher overall tolerance to otherwise lethal ethanol concentrations. Restoration of the NAD^+/NADH redox balance was the key to the ethanol tolerance of these mutants, facilitating higher glycolytic flux in the ethanol-compromised cells. Stimulation of glyceraldehyde-3-phosphate dehydrogenase activity leads to higher glycolytic flux in ethanol-stressed cells. This was likely achieved by increasing flux in the glycerol pathway to increase NADH turnover.

Stressors, such as ethanol and acetic acid affect various transport systems and modify the fluidity of the plasma membrane (Aguilera et al. 2006). Ergosterol, a component of yeast cell membranes that is essential to maintain fluidity and function of the cellular membrane, was directly correlated with the tolerance to ethanol and other stressors. The disaccharide trehalose was similarly shown to be important in maintaining membrane stability and preventing protein denaturation under stress. The importance and transient nature of trehalose accumulation during certain stress conditions have been well studied (Caspeta et al. 2015). Major genomic targets for adaptation to stressors include the adaptation of the cellular membrane, redox and ionic potentials, energy metabolism, and in some instances minor changes to protein structure. Rational engineering strategies for improved tolerances should thus consider the type and regulation of molecular responses following stress sensing, signal transduction, and activation of cellular functions in response to environmental stress. The cross-regulation between the different yeast responses due to various types of stress is paramount. This elasticity in the stress-signaling network will be beneficial to the generation of resistance to the multiple stresses inherent to 2G bioethanol production. Utilizing various "omics" data sets, adaptive evolution, and reverse engineering of robust phenotypes can contribute to the understanding how 2G stresses affect yeasts. Together with rational engineering, these approaches may contribute towards engineering robust *S. cerevisiae* strains for deployment in 2G processes.

Augmenting yeast capability with process development

The method of application of any yeast to a bioprocess for bioethanol production from pretreated and hydrolyzed

lignocelluloses will have a significant impact on the performance of the yeast in the process. Such bioprocess development aims to gain maximum benefit from the available/engineered inhibitor resistance of the yeasts, as mentioned in previous sections of this paper, to the extent that detoxification of lignocellulose-derived sugars is not required for industrial processes (Johansson et al. 2011). The objective is to maximize the utilization of the yeast's inhibitor resistance, without exceeding such capacity, since there is a correlation between fermentation ability and stress tolerance in *S. cerevisiae* (Johansson et al. 2011).

One of the first aspects of such bioprocess development to be considered is yeast pre-conditioning, which is a method of short-term adaptation to inhibitors that does not involve genetic modifications. Significant improvements to ethanol production in bioprocesses that utilised yeasts exposed to inhibitors during inoculum development have been demonstrated (Alkasrawi et al. 2006; Johansson et al. 2011; Zhang et al. 2014). Such pre-conditioning can also be achieved by the recycling of yeast from one cultivation to the next, since the recycled yeast will already be adapted/conditioned for fermentation performance in the presence of inhibitors (Zhang et al. 2014). Furthermore, the presence of preferred, often complex nutrients during the inoculum development and fermentation, was also found to improve the benefits derived from such pre-conditioning (Helle et al. 2004; Helle et al. 2008; Johansson et al. 2011). Although increasing the size of the yeast inoculum may increase biomass growth and tolerance, this may also have the adverse effect of reducing the ethanol yield (Helle et al. 2008; Zhang et al. 2014).

A second key process strategy is the application of fed-batch cultivation, where slow-feeding on an inhibitor-containing carbon source, will allow sufficient opportunity for the yeast to make optimum use of its in situ inhibitor detoxification/resistance phenotypes (Rudolf et al. 2004; Modig et al. 2008; Zhang et al. 2014; Sonego et al. 2018). The rate of feeding is a critical parameter for optimization of these cultures, to ensure that the metabolic and physiological capabilities for inhibitor resistance/detoxification are not exceeded, which will result in significant inhibition of the culture and loss of ethanol production (Zhang et al. 2014). Significant improvements in yeast performances were observed for slow fed-batch feeding, compared to once-off additions of the substrate (Modig et al. 2008). The application of online measurements of sugar concentrations represents a real-time method to track yeast responses to inhibitor-feeding and ensure that microbial capabilities for inhibitor resistance or detoxification are not exceeded (Taherzadeh et al. 2000; Nilsson et al. 2002). Such methods may prove invaluable in maximizing the process benefits derived from metabolic or genetic engineering of yeast for inhibitor resistance.

Conclusion and future prospects

Second-generation bioethanol production remains hampered by, among other factors, the sub-optimal fermentation capacities displayed by *S. cerevisiae*, when exposed to the microbial stressors inherent to this process. Microbial inhibitors, sub-par cellulolytic enzyme production as well as high temperatures, low pH, and nutrient starvation during fermentation present unique challenges to the inherent stress resistance mechanisms of *S. cerevisiae*. As such, stress modulation, and remediation via yeast development is a promising avenue, as various studies have undertaken to unravel the complexity of yeast stress responses with the goal of applying these mechanisms toward more robust whole cell bioconversion of LCB feedstocks. Advanced strain development using rational engineering, adaptation and reverse engineering have delivered promising *S. cerevisiae* strains. Single gene strategies or engineering of a small number of genes into strains have shown notable positive effects regarding several process relevant stress factors, as well as heterologous protein secretion. However, these strategies rely on prior knowledge of genes involved in the relevant response. Identifying a selection of genes linked to a polygenic phenotype is far more challenging. As such, empiric processes such as laboratory adaptive evolution and strain breeding approaches have been used to produce strains with superior stress tolerance phenotypes. Knowledge gained from such approaches has been used to reverse engineer genes or specific alleles into industrial strains to enhance specific phenotypes.

Advances in "omics" technologies can give a more holistic view from the inherent strain genetic potential to final endpoint phenotype. Elucidating these complex stress phenotypes allows for its modification towards set endpoints. Several studies have identified sets of genes and gene products that are upregulated under stress conditions with significant overlap between different environmental stresses. However, stress responses were also shown to be strain specific to a degree, an aspect that should be considered in reverse engineering strategies. Responses involved in tolerance to multiple process relevant stresses such as heat stress, redox balancing involved in lignocellulosic inhibitor tolerance, detoxification of ROS and improved energy management are imperative for superior tolerance, and therefore superior strains for 2G biofuel production. Knowledge gained from the identification of genes involved in these core responses in various strain backgrounds can make a significant contribution to developing superior strains for LCB conversion industries.

Strain breeding and studies using naturally isolated yeasts have shown that there is a wealth of genetic potential that remains to be unlocked in the pursuit for strains suitable for 2G bioprocesses in general and CBP in particular. Omics studies will be indispensable in unlocking this potential. Knowledge gained from synthetic biology projects such as

Yeast 2.0 is sure to further advance our understanding of stress modulation in yeast and will help inform the development of process ready organisms. Lessons learned through these platforms along with advances in genomic engineering strategies could finally unlock the potential of LCB as sustainable resource for fuels and chemicals. The biological capabilities of robust *S. cerevisiae* strains could be further maximised by adapting 2G process parameters to fully unlock the potential of 2G-bioethanol as a viable biofuel.

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Code availability N/A

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Declarations

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