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# Mitigating stress in industrial yeasts

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## CONTENTS

### 1. Introduction

### 2. Yeast stress

2.1 The major stress factors

2.2 Impact of stress on yeast

2.3 Assessment of yeast stress

2.4 Mitigation of yeast stress

2.5 Stressing yeast for industrial advantage

### 3. Case study – stress mitigation in Brazilian bioethanol yeasts

3.1 Overview of bioethanol production in Brazil

3.2 Microbial diversity in fermentations

3.3 Fermentation stress factors and their mitigation in Brazilian bioethanol

production

### 4. Conclusions

Acknowledgements

References

## Abstract

The yeast, *Saccharomyces cerevisiae*, is the premier fungal cell factory exploited in industrial biotechnology. In particular, ethanol production by yeast fermentation represents the world's foremost biotechnological process, with beverage and fuel ethanol contributing significantly to many countries economic and energy sustainability. During industrial fermentation processes, yeast cells are subjected to several physical, chemical and biological stress factors that can detrimentally affect ethanol yields and overall production efficiency. These stresses include ethanol toxicity, osmotic stress, nutrient starvation, pH and temperature shock, as well as biotic stress due to contaminating microorganisms. Several cell physiological and genetic approaches to mitigate yeast stress during industrial fermentations can be undertaken, and such approaches will be discussed with reference to stress mitigation in yeasts employed in Brazilian bioethanol processes. This article will highlight the importance of furthering our understanding of key aspects of yeast stress physiology and the beneficial impact this can have more generally on enhancing industrial fungal bioprocesses.

Key Words: yeast, fermentation, ethanol production, stress factors, *Saccharomyces cerevisiae*

## 1 Introduction

Yeasts, predominantly strains of the species *Saccharomyces cerevisiae*, represent the world's most important industrial microorganisms, being responsible for the production of a wide diversity of commodities in the beverage, food, industrial and pharmaceutical sectors. Yeast-derived alcohol is the most important product of fungal biotechnology, both volumetrically and economically speaking. During industrial alcohol fermentations, numerous environmental insults or stresses deleteriously affect activities of yeast cells and these include physical, chemical and biological factors that individually or collectively can reduce efficiencies of alcohol production. Examples of the more important stresses include high ethanol concentrations, glucose-induced osmotic pressure, pH extremes, temperature shock and metabolites produced by contaminating bacteria and wild yeasts. Specifically regarding yeasts employed for fuel alcohol (bioethanol) fermentations, such stresses can detrimentally affect the yields of ethanol, and this is of particular pertinence for sugarcane molasses processes in Brazil that will be discussed in this paper.

This paper will discuss approaches to mitigate stress during industrial fermentations, both at the physiological cell engineering and genetic improvement levels. Successful mitigation of environmental stress factors can benefit not only yeast alcoholic fermentations, but also other industrial bioprocesses that exploit fungi.

## 2 Yeast stress

### 2.1 *The major stress factors*

Many factors can cause problem fermentations conducted by yeast that result in poor metabolic activity. In the case of ethanol production processes, this is manifest by slow sugar conversion to alcohol (so-called "sluggish" fermentations) or, in more extreme cases, the result can be complete cessation of yeast metabolism (so-called "stuck" fermentations). Such

problems may arise due to poor fermentor parameter controls, poor nutritional composition of fermentation media, and the impaired physiological state of yeast cells. Regarding the latter, Table 1 outlines the major stresses that adversely affect yeast cell physiology during industrial fermentation processes. Of particular relevance for alcohol production, whether for beverages or biofuels, are ethanol toxicity, osmostress and extremes of pH and temperature. These factors can deleteriously affect yeast growth and metabolic activities, either as individual stresses, or more seriously when combined. For example, high ethanol concentrations (e.g. >10% v/v) when combined with high temperatures (e.g. >35°C) act synergistically greatly reducing yeast viability, especially toward the end of fermentation (Della Bianca et al. 2013).

Table 1 here

pH is also important, and although yeasts are acidophiles and will conduct efficient fermentations at a starting pH or between 5.0-5.5, if pH levels at the start of fermentation are below 4.0 this can result in sluggish fermentation performance, particularly due to the presence of organic acids (lactic and acetic acids) secreted by contaminating bacteria (Ingledeew, 2017a). High osmotic pressure, particularly in industrial media with high sugar (e.g. glucose) or salt (e.g. potassium) concentrations, can additionally impair fermentation performance by yeast (Cray et al., 2015; Medina et al., 2010).

Poor yeast nutrition can also be a cause of problem fermentations and this may include, in addition to low levels of fermentable sugars: free amino nitrogen deficiency, lack of oxygen, non-bioavailable minerals and vitamin depletion (Ingledeew 2017a). For second-generation bioethanol production processes that involve yeasts fermenting lignocellulosic hydrolysates, additional stressors can be problematic. For example, pretreatment-derived chemicals such as

weak acids (eg. acetic acid), furaldehydes and phenolic compounds can inhibit yeast metabolism and impair fermentation progress (Deparis et al., 2017).

Regarding biotic stress factors, the main ones pertinent to yeast alcohol fermentation processes are the presence of contaminating microorganisms such as lactic acid bacteria and wild yeasts (Ceccato-Antonini, 2018). The former can not only consume sugar that would otherwise be converted by yeast to ethanol, but also secrete lactic acid that can suppress yeast fermentative activities. Wild yeasts are problematic in beverage fermentations due to production of undesired off-flavours and aromas (Bokulich & Bamforth, 2017). In extreme cases, such yeasts may additionally produce killer toxins that are lethal to the production yeast strains (by acting as ionophores to disrupt plasma membrane functional integrity).

## 2.2 Impact of stress on yeast

In the face of environmental insults, whether chemical, physical or biological, yeast cells respond in multifarious ways in an effort to survive and thrive in the stressful situations. Ingledew (2017a) has discussed the impact of specific stresses encountered by yeasts in alcohol production process plants and Walker & van Dijck (2005) have reviewed the physiological and genetic responses of *S. cerevisiae* to stress. Table 2 summarises some of these responses.

Table 2 here

A lot of information has now been accrued, particularly at the molecular genetic level, into how *S. cerevisiae* species adapts to adverse environments. Yeast tolerance to stress is genetically complex and involves many signal transduction pathways (Ruis and Schüller,

1995; Walker, 1998a; Walker and van Dijck, 2005; van Voorst et al, 2006; Deparis *et al* 2017).

With regard to temperature stress, yeast cells respond by overproducing the disaccharide, trehalose that acts to stabilize yeast plasma membranes (Cray et al., 2015; de Souza et al., 2018). Other genes are upregulated in elevated temperatures, including those involved in ergosterol biosynthesis that also plays a role in conferring thermotolerance in yeast. During osmostress caused by high sugar or salt concentrations in fermentation media, yeast cells respond by over-producing the compatible solute, glycerol, which acts to protect cells from loss of intracellular water. Regarding ethanol, this yeast metabolite is toxic to yeast cells and at high concentrations can severely impact membrane structure and function (Walker, 1998a). Hallsworth (1998) has discussed how ethanol can induce a water stress response in yeast. Generally speaking, however, *S. cerevisiae* is regarded as a relatively stress-tolerant, or *robust*, yeast but there are limits to this tolerance. For example, temperatures beyond 35°C and ethanol levels in excess of 10% v/v would generally be expected to impair yeast physiology. Having said that, it is now possible, in controlled industrial fermentation processes to achieve ethanol yields above 20% v/v (Ingledew, 2017b; Walker & Walker, 2018). These high levels of ethanol are only achievable through proper yeast nutrition, especially nitrogenous and mineral nutrient bioavailability, together with temperature and pH optimization.

Overall, stress can deleteriously impact industrial yeast fermentations in a number of ways (Cray et al., 2015; Deparis et al., 2017). For example:

- Poor or ceased fermentation activity (i.e. sluggish or stuck fermentations)
- Decline in yeast viability
- Increased frequency of mutation
- Increased risk of microbial contamination

- Decreased number of re-pitchings (brewing yeast)
- Altered yeast flocculation
- Increased production of glycerol
- Excretion of protease (by brewing yeasts to reduce beer foam stability)
- Production of undesired flavour/aroma compounds in fermented beverages

### *2.3 Assessment of yeast stress*

A range of analytical methods, some relatively straightforward and others more technologically sophisticated, can be employed to measure the impact of stress on yeast physiology and viability (Walker, 2012). Regarding the former methods, light and fluorescence microscopy can be used to directly visualise stressed yeast and when using vital stains this can also provide quantitative information on yeast viability. For example, bright-field dyes or fluorophores such as methylene blue or propidium iodide, respectively, can indicate viability changes in yeast following stress. Table 3 summarises some microscopy-based methods to assess yeast stress.

Table 3 here

For microscopic brewing yeast viability assessments, these are routinely carried out in industry using methylene blue or violet with a haemocytometer and results quoted as percentage viability (Smart et al., 1999). The use of flow cytometry can greatly facilitate the evaluation of stress effects in millions of individual yeast cells in a rapid manner and this has proved particularly useful for brewing yeast populations (e.g. Boyd et al., 2003).

Capacitance-based methods can also be used to rapidly assess yeast cell viability, and have proved especially beneficial in the brewing industry to accurately monitor and control the pitching rates (inoculum cell density) of viable cells into fermenters (Thiele and Back, 2012).

Such methods utilise the fact that viable cells with intact membranes build up an electrical charge when subjected to radio frequency, but dead cells (with damaged membranes) do not. Other technologically advanced methods are available to assess yeast stress. For example, developments in yeast transcriptomics can facilitate rapid profiling of individual stress-responsive gene expression during industrial fermentation (Higgins et al., 2003). In this context, De Nicola et al. (2007), Verbelen et al. (2009) and Yoshikawa et al. (2008) have employed genome-wide microarrays to identify over- and under-expressed genes in *S. cerevisiae* in response to stress caused by zinc-limitation, oxidative stress and ethanol toxicity, respectively.

#### 2.4 Yeast stress mitigation

Walker & Walker (2018) have reviewed some of the ways in which yeast stress can be alleviated to benefit alcohol fermentations in general. Specifically with regard to stressful effects on wine yeasts, Specht (2015) has reviewed ways to mitigate stuck fermentations of grape must. Table 4 summarises some practical approaches of stress mitigation in industrial yeasts.

Table 4 here

Importantly, choosing the correct yeast strain employed for particular industrial processes is paramount since some strains of *S. cerevisiae* are inherently more stress-tolerant than others. For example, isolation of naturally occurring stress-tolerant yeasts from environmental sources, including industrial processes such as bioethanol plants (see Basso et al., 2008 and section 4) can prove advantageous for particular yeast fermentations. The tolerance of existing yeast strains can be improved via several physiological and genetic strategies. Yeast

stress-protection strategies based on cell physiology include careful control over nutrient bioavailability either during fermentations or by preconditioning seed cultures prior to fermentor inoculation (Walker and Walker, 2018). However, more directed methods to improve the inherent stress-tolerance of yeast cells include *adaptive evolution*. This has proved successful to generate strains able to withstand several environmental stress factors (Deparis et al., 2017). For example, temperature and ethanol stress resistance in yeast can be achieved by prolonged serial culture of cells at elevated temperatures (e.g. Caspeta et al., 2016), or with higher concentrations of ethanol, respectively. Chemostat cultivations are particularly advantageous in this respect, as originally described by Brown and Oliver (1982) to introduce ethanol tolerance traits in yeast strains. Because ethanol tolerance in yeast is a polygenic trait (van Voorst *et al*, 2006), genetic modification may not be a profitable approach to mitigate ethanol toxicity in yeast. Ethanol is toxic to yeasts due to cell membrane damage, so physiological methods aimed at protecting cell membrane structural and functional integrity have proved beneficial. For example, Walker and Maynard (1997); Walker (1998b); Birch and Walker (2000) and Trofimova *et al*, (2010) have shown that magnesium ions exert membrane-protective effects in ethanol-stressed yeast cells. Similarly, Stanley *et al* (2010) and Lam *et al* (2014) have shown that maintaining K<sup>+</sup> ion balance across yeast cell membranes mitigates ethanol stress in yeast and this may enhance fermentative activity. Regarding magnesium, this metal ion has been shown to counteract other stressors besides toxic levels of ethanol, including heat-shock and ultrafreezing (Walker and Birch, 1999).

With regard to mitigating the effects of high osmotic pressure on yeast cells, particularly caused by high concentrations of glucose, industrial alcohol producers employ simultaneous saccharification and fermentation (SSF). SSF processes are commonplace in large fuel

alcohol distilleries that employ cereal starch feedstocks (Pilgrim, 2017). For example, in corn bioethanol plants, saccharifying amylolytic enzymes such as glucoamylase are active during the fermentation to prevent build-up of glucose that would otherwise cause osmotic stress to the yeast cells. Osmotic stress on yeast results in an over-production of glycerol which detracts from the yields of ethanol, and it is also possible to reduce yeast biosynthesis of glycerol by deleting genes for the enzymes converting dihydroxyacetone phosphate to glycerol .

Weak acid inhibition of yeast growth during fermentation can also be mitigated. For example, by reducing acetic acid to ethanol using NADH-dependent reactions this can help to alleviate inhibition lignocellulosic hydrolysate fermentations for second-generation bioethanol production (Medina et al., 2010).

In addition to physicochemical stress effects on yeast, biotic factors are also important. For example, the presence of unwanted, or contaminant, microorganisms in yeast-based industrial processes can present serious fermentation efficiency problems linked to yeast stress. This is manifest either by direct competition by contaminants for nutrients, by the presence of inhibitory microbial metabolites or by direct predation. Many bacterial contaminants exist in industrial yeast fermentations, with various species of the *Lactobacillus* genus being commonplace. Hygienic practices in yeast-based industries (e.g. breweries, wineries, distilleries, yeast production plants and biorefineries) represents the best approach to control contaminant microbes and these have been discussed by Richards (2017) in relation to alcohol production plants. Control of commonly encountered bacterial contaminants (lactic acid and acetic acid bacteria) using antimicrobial agents in yeast alcohol production has been discussed by van Zyl and Kauer (2017), but wild yeast contaminants in yeast fermentations are not so easy to control (Abbott and Ingledew, 2009).

In addition to mitigating stress on yeast cells through the control of contaminating microbes, and though cell physiological approaches discussed above, genetic approaches to improve yeast strain stress tolerance may also be employed. Classical yeast genetics involving hybridization, protoplast fusion, rare mating and mutagenesis has proved successful in constructing new *S. cerevisiae* hybrids with enhanced stress tolerance (eg. Gonzalez-Ramos *et al*, 2016). However, one dilemma in using conventional yeast genetic improvement strategies is the complex genetic background of industrial strains of *S. cerevisiae*. These are often aneuploid or polyploidy in nature (Bokulich and Bamforth (2017) making improvement of existing strains relatively imprecise and can result in genetic instability of resultant modified strains. In contrast, strain engineering via recombinant DNA technology or gene editing procedures circumvents this dilemma and has proved beneficial to improve stress tolerance in yeasts. Such approaches to improve yeast alcohol fermentations have been reviewed by Mapelli (2014), Deparis *et al.* (2017) and Walker and Walker (2018). These procedures aim to construct new yeast strains with improved traits that include “robustness” and enhanced industrial fermentation performance. Gene editing technologies involve very targeted gene deletions and insertions and represent very attractive alternatives to more conventional recombinant DNA approaches of yeast strain improvement. Many of these are based on CRISPR (Clustered Regularly Interspaced Palindromic Repeats) and CRISPR-associated protein-9 nuclease (Cas9) which can greatly improve yeast strain characteristics. (Jamal, 2017). Conferment of yeast stress tolerance using CRISPR/Cas9 gene editing technology include the following examples:

- high ethanol tolerance (Swinnen *et al.* 2012),
- acetic acid tolerance (Meijnen *et al.* 2016; Si *et al.* 2017)
- thermolerance (Yang *et al.* 2013).

Another synthetic biology approach to construct designer yeast strains involves SCRaMbLE (or Synthetic Chromosome Recombination and Modification by *LoxP*-mediated Evolution). Use in conjunction with adaptive evolution, SCAMBLE can be used to rapidly identify new stress-tolerant yeast strains (Walker and Walker, 2018). Modern strain engineering approaches to mitigate yeast stress in alcohol fermentations that utilise gene editing and synthetic biology, have been discussed by French (2009) and Walker and Walker (2018).

However, an important aspect regarding the development and exploitation of genetically manipulated (GM) yeasts relates to regulatory approval issues. In this respect, cisgenic methods, or *self-cloning*, of yeast strains to improve stress tolerance may be more attractive than transgenic recombinant DNA technology methods as discussed by Argyros and Stonehouse (2017). Specifically, a commercial GM strain of *S. cerevisiae* has now been developed that secretes a heterologous glucoamylase that enables starch saccharification in SSF processes. This reduces dependency on exogenous amylolytic enzymes whilst also reducing glucose-induced osmotic stress on yeast (Argyros and Stonehouse, 2017). Other engineered yeast strains have been constructed with an alternate electron acceptor to glycerol resulting in fermentations with higher yields of ethanol and concomitant lowered levels of glycerol. Overall, the introduction of genetically-enhanced yeasts able to withstand the rigours of large-scale bioethanol fermentations can dramatically improve industrial fermentation efficiency. In fact, their commercial introduction has been highly successful and represents the largest deployment of such microorganisms in industry. Looking ahead, the construction of yeasts with entirely synthetic genomes conferring wide stress tolerance is on the horizon.

#### 2.4 Can we stress yeast for industrial advantage?

It is well established that if yeast cells are subjected to a sub-lethal stress, such as a transient heat-shock, then cells will subsequently acquire tolerance to a further stressor that would otherwise be lethal (Hohmann and Mager, 2003). That is, the main function of such adaptive stress responses in yeast is to enable cells to survive and grow under stressful conditions. This is primarily due to the accumulation of intracellular trehalose, which acts as a membrane stabilizing molecule and has been termed a “general stress metabolite” in yeast (Walker, 1998a; Trevisol et al. 2011). There are several examples of exploitation of adaptive yeast stress responses for industrial advantage, and these are summarised in Table 5.

Table 5 here

### **3 Case study – stress mitigation in Brazilian bioethanol yeasts**

#### *3.1 Overview of bioethanol production in Brazil*

Brazil is the largest producer of bioethanol (fuel alcohol) from sugarcane in the world (Andrietta et al. 2007; Della Bianca et al. 2013; Gombert and van Maris, 2015; Basso et al. 2019). The fermentation substrate may either be the raw cane juice or the molasses derived from sugar refining. The process starts with sugarcane being pressed to separate the sugar-containing broth (sugarcane juice) from the fibrous solid residue (bagasse). Raw sugar, containing sucrose crystals, are obtained by concentration and crystallization of the sugarcane broth. The residue of this step is a dark and viscous sucrose-rich material, named molasses. Molasses can be mixed with either water or sugarcane juice for preparation of the must and used for fermentation, either in a fed-batch or in a continuous process, both operated with yeast cell recycle (Amorim et al. 2011; Basso et al. 2011; Della Bianca et al. 2013).

In general, fermentation starts by the addition of the sugarcane based-must, that contains 18–22% (w/v) sugars, of a high-density yeast cell suspension, which represents 30% of the vessel volume. In the fed-batch process, feeding lasts 4–6 h, and the fermentation is completed within 10–12 h. At the end of the fermentation, ethanol titres between 8–12% (v/v) are achieved, with a final yeast cell density of 10–14% (w/v, on a yeast cell wet basis). Thereafter, the yeast cells are separated from the fermented broth (referred as to as wine) by a continuous centrifugation step. The centrifuged yeast slurry is conveyed to separate tanks where it is diluted with water and treated with sulphuric acid (to pH 1.8–2.5) to reduce bacterial contamination. This process is known as acid-washing and it lasts for 1–2h. The yeast-free fermented broth is subsequently distilled for ethanol purification. After the end of the acid-washing step, yeast cells are reused in a subsequent fermentation cycle. A schematic view of the process is depicted in Fig. 1.

Fig. 1 here

The process configuration presented in Fig. 1 utilises very-high yeast cell densities and operates with intensive yeast cell recycle. In doing so, this process is rather peculiar as it allows up to two fermentation cycles each day, lasting for almost 250 days (the duration of the sugarcane harvest season). It is important to mention that the reuse of yeast cells reduces considerably the need for yeast propagation. Therefore, it results in very minor conversion of the substrate carbon source into yeast cell biomass as compared to other ethanol fermentation processes (Della Bianca et al. 2013; Basso and Lino 2018).

### *3.2 Microbial diversity in fermentations*

In Brazilian bioethanol production, the microbial diversity in the fermentation tanks is composed basically by diverse yeasts and bacterial strains due to the non-aseptic nature of the process (Basso et al. 2008). As reported by the seminal work performed by Basso et al (2008), a “great biodiversity was observed in industrial fermentations, each distillery with its own population” of *S. cerevisiae* strains. According to these authors, prevalent and persistent strains were identified, and these strains were synonymous with competitiveness and stress tolerance, respectively, during industrial fermentation.

In contrast, the intrinsic bacterial population is regarded as a major drawback during industrial ethanol production. In addition to the fact that these strains deviate feedstock sugars away from ethanol formation, there are also various detrimental effects of bacterial metabolites that act upon the fermenting yeast, leading to reduced ethanol yield, yeast cell flocculation, foam formation and decreased yeast viability (de Oliva-Neto and Yokoya 1994; Basso et al. 2014; Basso and Lino 2018). Bacterial-induced yeast flocculation impairs the centrifuge efficiency and decreases productivity due to mass transfer issues (Basso et al. 2008). Excessive foam formation may increase operational costs due to the intensive use of antifoam chemicals (Nielsen et al., 2017).

As reported previously by laboratory culture-dependent methods (Costa et al. 2008; Lucena et al. 2010), following research reported by Gallo (1989), it has recently been verified, using culture-independent methods, accessed through 16S rDNA gene sequencing, that the vast majority (70 to 99 %) of the genome sequences found in fermentation tanks in ethanol industrial plants are affiliated to the *Lactobacillus* genus (Costa et al. 2015; Bonatelli et al. 2017). It has been concluded that each distillery appears to have a distinct microbiome and that these communities persist over time (Bonatelli et al. 2017). Table 6 highlights the microbial diversity in Brazilian bioethanol fermentation processes.

Table 6 here

In terms of yeast contaminants, apart from the indigenous strains of *S. cerevisiae* (Basso et al. 2008), contamination episodes in distilleries from the North-eastern region of Brazil has been reported to result in the identification of *D. bruxellensis*, *Candida tropicalis*, *Pichia galeiformis*, and *Candida* spp. as the main yeasts present in fermentation tanks (Basílio et al. 2008). *D. bruxellensis*, which is considered to be the most serious contaminant yeast species (Liberal et al. 2007), was not detected using culture-independent methods in sugarcane and mixed juices from a distillery of the Centre-West region of Brazil (Costa et al. 2015). Cabrini and Gallo (1999) have found *Candida*, *Torulopsis*, *Pichia* and *Schizosaccharomyces* as contaminating non-*Saccharomyces* yeasts in a distillery located in the Southeast region of Brazil.

### 3.3 Fermentation stress factors and their mitigation in Brazilian bioethanol production

#### 3.3.1 Metal ions

The presence of toxic levels of aluminium in sugarcane-based industrial substrates is responsible for decreasing fermentation performance. Due to the acidic condition of fermentation, aluminium (absorbed by sugarcane in acid soils) is mainly present as its toxic form (i.e. the three-valent ion  $Al^{3+}$ ). Aluminium, therefore, is considered highly toxic under acidic conditions, being particularly deleterious to fermenting yeast cells. Their effects include reduction in yeast cell viability and in ethanol yield, as well as a sharp decrease in cellular trehalose content (Basso et al. 2004). It has been shown that such deleterious effects can be partially alleviated by addition of magnesium ions into the wort. This is further evidence of a stress-protective role for magnesium in yeast fermentations, as discussed previously in this article. Moreover, such negative effects can be completely abolished by

enriching with molasses sugarcane juice-based musts, which is believed to be related to a chelating property exerted by the former substrate (Basso et al., 2004). Another way to mitigate aluminium toxicity is the use of specific robust industrial yeast strains that differ from traditional ones regarding aluminium tolerance. These strains exhibit higher fermentation performance when compared to commercial baker's yeast strains of *S. cerevisiae*, or even with commonly used selected yeast strains. This is manifest by their ability to maintain higher cell viability, higher ethanol yield and lower aluminium cell accumulation in media containing this toxicant. For example, Brazilian yeast strain isolate *S. cerevisiae* CAT-1 has been reported to be less sensitive in comparison to another isolate from Brazil, PE-2, and to commercial baker's yeast (Basso et al., 2011).

Toxic levels of cadmium have been also reported to accumulate in yeast cells during cell recycle, leading to lower cell viability, decreased intracellular trehalose content, and reduced ethanol yield when compared to non-accumulated cadmium cells. The use of molasses-rich medium or the use of vinasse were reported to alleviate cadmium toxic effects (Mariano-da-Silva and Basso 2004).

### 3.3.2 Modulating substrate composition

Brazilian ethanol plants may conduct fermentations with different sugarcane must compositions. Plants that are attached to sugar factories usually ferment sugarcane musts composed of by-product molasses diluted with water or a mix of sugarcane juice and molasses, whereas autonomous distilleries process only concentrated sugarcane juice, rather than molasses (Lopes et al. 2016). Although sugarcane-based fermentation media have successfully been used for ethanol production for decades, they do represent challenging conditions for the fermenting yeast *S. cerevisiae*. Apart from the nutrients present in industrial sugarcane-based media, this fermenting broth also contain inhibitors which can be

feedstock- or process-related (Della Bianca et al. 2013; Basso and Lino 2018). During the heating step of sugarcane juice sugar-degradation products are formed, and such compounds can act as fermentation inhibitors. Examples are furaldehydes (e.g. furfural) and Maillard degradation products (e.g. melanoidins) (Eggleston and Amorim 2006).

Interestingly, Stambuk et al. 2009 identified that Brazilian fuel ethanol strains showed amplifications of the telomeric *SNO* and *SNZ* genes. These genes are involved in the biosynthesis of vitamins B6 (pyridoxine) and B1 (thiamin), respectively. According to the authors, these amplifications provide an important adaptive advantage under the substrate conditions in which the yeast are propagated. In addition, Santos et al. 2017 evaluated how industrial fuel ethanol strains compete for nutrients in industrial-like fermentation conditions. They have demonstrated, via quantitative proteomics, that proteins involved in response to oxidative stress and trehalose synthesis are associated with better fermentation performance in pairwise competition experiments.

A very high gravity (VHG) repeated-batch fermentation system using an industrial strain of *S. cerevisiae* (PE-2) was successfully operated during fifteen consecutive fermentation cycles, using an operational strategy called “biomass refreshing step”, in which fresh yeast were removed from each recycling step when viability was lower than 50%. This strategy prevented decreases on yeast viability and promoted accumulation of intracellular storage carbohydrates (Pereira et al. 2012). However, it is important to mention that the previous work was performed with a glucose-based medium supplemented with corn steep liquor. Recently, the utilisation of VHG conditions in sugarcane juice fermentations, using 30 °Brix and cell recycle, showed industrially-relevant ethanol yield and productivity, coupled to the maintenance of high yeast viability, when the fermenting broth was supplemented with 16 mM urea (Monteiro et al. 2018). Finally, according to Abreu-Cavalheiro and Monteiro (2013), there is a lack of knowledge of genes that are important in conferring stress resistance

during the cell recycling process. Such knowledge should guide further improvements in industrial strains via metabolic engineering approaches.

### 3.3.3 Combating Bacterial Contamination

In view of the findings mentioned above concerning bacterial contamination, it has been suggested by Basso et al (2014) that distilleries in Brazil invest in methods that specifically control *Lactobacillus* spp. rather than any other bacteria. In fact, Basso et al. (2014) stated that in industrial sugarcane plants even more species-specific approaches should be adopted for effective control of bacterial contamination due to differential effects of lactobacilli in such fermentations. Traditionally, when bacterial contamination is not properly controlled with the classical sulphuric acid wash-treatment during the yeast cell recycle step, Brazilian distilleries attempt to control bacteria with antibiotics (Ceccato-Antonini 2018). However, there is a relative lack of systematic investigation on the effects of the acid-wash treatment on the fermenting yeast. One of the few works on this topic have demonstrated that genes involved in energy production (e.g. glycolysis, the tricarboxylic acid cycle, the electron transport chain, fermentation and aerobic respiration) are down-regulated, whereas genes related to protein synthesis are up-regulated in the acid-wash phase (Brown et al. 2013). Although *S. cerevisiae* generally can tolerate low pH, the sulphuric acid treatment (pH 1.5) results in physiological perturbations on yeast cells. These include mineral (N, P, K, Mg) leakage, decreasing levels of cellular trehalose content and reduction in cell viability (Ferreira et al., 1999). Interestingly, the Brazilian industrial PE-2 yeast strain displays a higher fitness in low pH when compared to a laboratory strain (CEN.PK113-7D) and to a commercial bakers' strain (Della-Bianca et al. 2014), as well as to the reference laboratory strain S288c (Argueso et al. 2009). In addition, industrial yeast strains that tolerate the stressful conditions of industrial fermentations (Della-Bianca and Gombert 2013), particularly during the acid

wash step, are known to present higher cellular trehalose levels (Basso et al. 2008) and increased resistance towards oxidative stress (Argueso et al. 2009).

To circumvent the problem of inefficient bacterial contamination control by acid-washing, antibiotics have been used, including penicillin, streptomycin, and tetracycline, with monensin and virginiamycin being the preferred ones (Dellias et al. 2018). Monensin is a carboxylic ionophore antibiotic that affects cation channels and glycolysis (Delort et al. 1989), while virginiamycin is a streptogramin antibiotic that inhibits protein synthesis at the ribosomal level (Pechère 1996). Both of these agents exhibit antimicrobial activity against gram-positive bacteria, such as lactobacilli (Newbold and Wallace 1988; Walter et al. 2019). However, there are increasing concerns about the potential emergence of antibiotic resistance and their residual effects in the environment and in distillery by-products (such as inactive dried yeast for animal feeding) (Braga et al. 2017).

The development of other antimicrobial products has significantly reduced bacterial contamination in the fuel alcohol industry. This has been necessary due to pressures to reduce antibiotic usage, mainly by distilleries producing animal feed co-products. Chlorine dioxide and hop acids derivatives (alpha and beta fraction) are among the new antimicrobials used (Ceccato-Antonini 2018). Natural products such as hop acids, propolis and chitosan have been reported to be effective in controlling bacterial contamination in fuel ethanol fermentations (van Zyl and Kauers, 2017).

An interesting strategy to further reduce bacterial contamination without the use of antibiotics is to increase the ethanol titre during the acid treatment. This procedure was verified by Costa et al. (2018) to cause the complete loss of bacterial cell viability, without affecting ethanol yield and yeast cell viability.

### 3.3.4 Fermentation temperature control

Although in industrial-scale ethanol production, the fermentation temperature is maintained by using cooling systems, heat generated by yeast metabolism and/or high environmental temperature can raise the fermentation temperature beyond an optimal range. Heat stress is therefore another important stressor encountered by yeast cells (Deparis et al., 2017; de Souza et al. 2018; Burphan et al. 2018).

Due to high ethanol titres towards the end of each fermentation cycle [8-12 % (v/v)], ethanol stress is also a major concern in yeast fermentations in Brazilian distilleries (Basso et al. 2011; Amorim et al. 2011; Cray et al. 2015). Causative alleles (*MKT1* and *APJ1*) were identified in a Brazilian bioethanol production strain (VR-1) with a clear effect on high ethanol tolerance (Swinnen et al. 2012). The inhibitory effects of ethanol on *S. cerevisiae* is still not completely understood. Nonetheless, fermentations that produce broths with even higher ethanol titres are highly desirable, since this reduces substantially water consumption and energy expenditure in the subsequent distillation step. In addition, such conditions would favour the energy balance of the process as a whole and it would also improve its sustainability (Lopes et al. 2014). Therefore, enhancing ethanol tolerance by yeasts is an important goal for stress mitigation. A substantial increase in ethanol tolerance was reported by simply increasing extracellular potassium levels and pH in the culture medium in industrial-like conditions (Lam et al. 2014). Furthermore, it was demonstrated that overexpression of only two genes (*TRK1* and *PMA1*) related to potassium- and H<sup>+</sup>-transporters were sufficient to increase ethanol titre and ethanol tolerance during ethanol fermentations.

Ethanol and temperature effects on yeasts are known to be closely related as ethanol becomes increasingly toxic to growth and viability at higher temperatures (Nagodawithana et al. 1974; Casey et al. 1984). As a way to mitigate the ethanolic stress in Brazilian industrial fermentations, a process known as Ecoferm<sup>®</sup> proposes the use of specially designed chillers

to keep fermentation temperature below 30 °C. This technology has claimed to achieve ethanol titres as high as 16 % (v/v) in fermentation cycles that last for 17 h (Fermentec, 2019). An important key technology that is already implemented in industrial scale is known as Altferm<sup>®</sup>. By using more rigorous process control and monitoring strategies and customized robust yeast strains, the process is able to deliver final ethanol titres of 12 % (v/v), without the requirement of costs associated with the use of extra-cooling technologies. A key factor to increase the ethanol concentrations has been the selection and use of robust yeast strains (Lopes et al. 2016; Fermentec, 2019). The increase in ethanol titres, and as a consequence, the decrease in vinasse production, is considered a holy grail to the Brazilian ethanol industry. It is estimated that for each unit percentage increase in the final ethanol titre, there is a considerable reduction in the volume of vinasse produced, in addition to steam power and water savings.

Adaptive laboratory evolution has been used to generate new yeast strains with superior tolerance towards temperature (Caspeta et al. 2019). It was observed an upregulation of *PMA1* transcription and the downregulation of its negative regulator *HSP30*, increased tolerance towards osmotic and acidic stresses. This, however, was followed by a reduced ethanol tolerance in the evolved strain, possibly caused by an exacerbation of proton efflux. Such strategies remain to be tested for yeast strains isolated from the Brazilian process (de Souza et al. 2018; Basso et al. 2019).

### 3.3.5 Customized yeast strains

Similarly to developments in the wine industry discussed by Pretorius and Bauer (2002), where customized wine yeast strains have been introduced for improved fermentation performance, processing efficiency and biological control of wine-spoilage microorganisms,

customized fuel ethanol yeast strains are appearing on the market. The Belgium-Brazilian company Global Yeast is offering yeast strains (Excellomol) designed specifically for high-gravity cane molasses fermentation (Global Yeast 2019). Another interesting example is the case of the Sucramax<sup>TM</sup> yeast strain offered by Lallemand/Mascoma which has been claimed to deviate less carbon to glycerol formation with a concomitant increase in ethanol yield during sugarcane fermentation (Argyros and Stonehouse 2017). A very robust yeast strain (Safdistil<sup>TM</sup> C-70) is claimed by the French yeast company, Leaf/Lesaffre, to be able to ferment molasses at temperatures as high as 35°C (Lesaffre 2019). Finally, the Brazilian company Fermentec proposes the use of yeast monitoring in industrial fermentations as the basis for the selection of customized strains according to the peculiar characteristics of each distillery, aiming for increased yield and productivity, as well as better process control. According to this company, various distilleries have been using what has been called Tailored Yeast Strains<sup>®</sup> (Lopes et al 2016).

#### 4 Conclusions - industrial implications

Table 7 summarises some of the approaches to mitigate stress in industrial yeasts that will lead to improved fermentation performance. These approaches should be more generally applicable to other fungal bioprocesses.

Table 7 here

Finally, it is important for yeast technologists to monitor signs of stress effects on their production strains and there are several straightforward and technologically sophisticated methods available (see Section 2.3). If yeast stress is judged to be a reason for reduced performance of an industrial process, then measures should be undertaken to mitigate such stresses. Some of these measures are based on understanding of yeast nutritional physiology,

whilst others involve strain engineering to generate new yeast strains with enhanced stress tolerance and improved industrial productivity.

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Table 1 Stress factors impacting physiology of industrial yeasts  
(Adapted from Walker & Walker, 2018)

<b>Type of stress</b>	<b>Examples</b>
Physical	Temperature shock (hot or cold), osmotic stress, anhydrobiosis, aerobiosis/anaerobiosis, hydrostatic/gaseous pressure, G-forces, mechanical shear stress, irradiation
Chemical	Ethanol and other toxic metabolites (eg. acetaldehyde), CO <sub>2</sub> , extraneous chemical inhibitors, nutrient limitation/starvation, oxidative stress (reactive oxygen species), pH fluctuations, metal ion toxicity, chemical mutagens
Biological	Cellular ageing, genotypic changes (spontaneous and induced mutations), microbial competition, yeast killer toxins

Table 2. Examples of responses of yeast cells to stress (Adapted from Walker 1998a; DeParis et al., 2017)

<b>Stress response</b>	<b>Comments</b>
Cell division cycle arrest	Many stressors will arrest the yeast cell cycle, and prevent further growth
Apoptosis	Toward the end of their finite budding lifespan, senescent cells enter apoptosis
Induction of heat/cold shock protein synthesis	Several heat-shock proteins are induced in response to temperature and other stresses
Induction of stress enzymes	For example, antioxidant enzyme induction (eg. superoxide dismutase and catalase) due to oxidative stress
Cell membrane structural changes	Disruption of membrane integrity is caused by several stressors
Genotypic changes	Petite mutations are common in brewing yeasts subjected to stress
Induction of trehalose biosynthesis	Cells over-produce trehalose in response to heat-shock and other stressors
Induction of glycerol biosynthesis	Being a compatible solute, glycerol is over-produced in response to osmostress
Changes in intracellular metal ion homeostasis	Several stressors result in loss on key metal ions such as Mg and Zn

Table 3 Assessment of yeast stress using microscopy

<b>Type of microscopy</b>	<b>Basis of method</b>	<b>References</b>
Light microscopy	Dye reduction using methylene blue or violet is employed to assess the viability of industrial yeasts  Visualisation of yeast cellular morphology as “quartets”	Smart et al., (1999)  Lodolo & Cantrell (2007)
Confocal microscopy	Imaging of intracellular changes in stressed yeast cells	Schlee et al (2006)
Fluorescence microscopy	Several fluorophores can be used to assess aspects of yeast stress physiology. For example: <ul style="list-style-type: none"> <li>- Live/dead differentiation (MgANS, DiSBAC, Fun-1)</li> <li>- Membrane lipid content (Nile red)</li> <li>- DNA ( Propidium iodide, DAPI)</li> <li>- Glycogen content (Acraflavin)</li> <li>- Trehalose content (ConA FITC)</li> <li>- Aged cells/bud scars (Calcoflour)</li> <li>- Zinc content (Newport Green, Fluo-Zn3, Rhodo Zn-1)</li> <li>- Oxidative stress (Luminol, OxyBurst Green)</li> </ul>	Powell (2005); Thiele & Back,(2012); Walker (2012)
Multi-photon Scanning Fluorescence Microscopy	Assessment of membrane fluidity in individual yeast cells	Learmonth & Gratton (2002).
Scanning electron microscopy	Surface visualisation of stressed yeast cells	Birch & Walker (2000)
Atomic force microscopy (AFM)	AFM gives nano-scale resolution of live yeast cell surfaces and can provide quantitative information on cell wall topology, roughness, adhesion, flocculation and yeast-yeast interactions.in cells subjected to various stresses.	Adya et al. (2005); Canetta, Walker & Adya (2006); Canetta., Walker & Adya (2009)

Table 4 Practical measures to mitigate stress in industrial yeasts  
(adapted from Walker & Walker (2018))

<b>Stressor</b>	<b>Stress mitigation strategy</b>
General stress	Use inherently stress-resistant or adaptively evolved or genetically engineered yeast strains
Temperature (eg. heat-shock)	Tight control over fermentation temperature (cooling)
Ultrafreezing (-196°C)	Suspending cells in Mg salts will protect yeast cells
Osmostress (glucose-induced)	Use of simultaneous saccharification and fermentation
Ethanol toxicity	Ensure correct yeast nutrition. Supply oxygen for membrane sterol biosynthesis. Removal of fermentation alcohol using vacuum fermentations
Anaerobiosis	Pre-oxygenation of yeast or microaeration during fermentation
Excess acidity or alkalinity	pH control during fermentation
Microbial competition	Hygienic practices or use of antimicrobial agents

Table 5 Examples of stressing yeast for industrial applications

<b>Applied stressor</b>	<b>Yeast stress response</b>	<b>Industrial application</b>
Heat-shock	Yeast cells with elevated trehalose levels following heat shock are subsequently more resistant to stress (e.g. freeze-resistance).	Baker's yeast for frozen dough baking applications
UV-irradiation	UV-irradiated yeast cells will convert ergosterol in yeast membranes to ergocalciferol (vitamin D2)	Vitamin D-enriched yeast for baking and for nutraceuticals
Osmotic shock	Lowering osmotic potential of yeast growth media (eg. with NaCl or sorbitol) will elevate cellular trehalose and glycerol levels	Potential application of stress tolerant yeasts in fermentation processes
Oxidative stress	Induction of stress-responsive enzymes (superoxide dismutase, catalase), but also stimulation by oxygen of membrane sterol and unsaturated fatty acid synthesis	Yeasts oxygenated or aerated prior to alcoholic fermentation will be more stress-tolerant due to membranes enriched in ergosterol and oleic acid
Heat plus salt	Autolysis	Production of yeast extracts and yeast beta-glucan

Table 6 Microbial diversity, with emphasis on bacterial contaminants, present in Brazilian ethanol fermentation vats

<b>Sampling location and Identification technique</b>	<b>Isolates (most prevalent)</b>	<b>Reference</b>
Samples of concentrated yeast cell suspension, culture-dependent method (MRS broth)	<i>L. fermentum</i> (15.04%), <i>L. helveticus</i> (14.08%), <i>L. plantarum</i> (5.69%), <i>Bacillus coagulans</i> (15.09%), and <i>B. stearothersophilus</i> (6.91 %)	Gallo (1989)
Strains isolated from fermented wine from various distilleries, culture-dependent method (MRS broth)	<i>L. fermentum</i> , <i>L. plantarum</i> , <i>L. paracasei</i> , <i>L. mesenteroidis</i> , etc	Costa et al. (2008)
Sampling in fermentation tanks, culture-dependent method (MRS broth)	<i>L. fermentum</i> and <i>L. vini</i>	Lucena et al. (2010)
Fermented wine from fermentation tanks. Pyrosequencing-based profiling of bacterial and archaeal 16S rRNA genes and the fungal internal transcribed spacer region	<i>Lactobacillus</i> spp. and unclassified Lactobacillaceae (Some bacterial strains might have been removed during the centrifugation step prior analysis)	Costa et al. (2015)
Sampling in fermentation tanks, culture-independent method (bacterial 16S rDNA sequencing)	<i>Lactobacillus</i> spp., <i>Weissella</i> spp., <i>Pediococcus</i> spp., <i>Acetobacter</i> spp., and <i>Anaeosporobacter</i> spp.	Bonatelli et al. (2017)

Table 7 Summary of yeast stress mitigation approaches

<b>Stress mitigation approach</b>	<b>Comments</b>
Ensure correct yeast nutrition	Balance of minor nutrients, metal ion bioavailability
Control fermenter parameters	Temperature, dissolved oxygen, pH, agitation, media specific density
Yeast physiological conditioning	Correct cool storage of yeast. Optimum pre-propagation conditions (eg. Avoidance of acid-washing)
Microbial contamination control	Hygienic operation of yeast fermentation processes, and use of antimicrobial agents when necessary
Use of improved yeast strains	Physiological and genetic methods to enhance resistance to stress

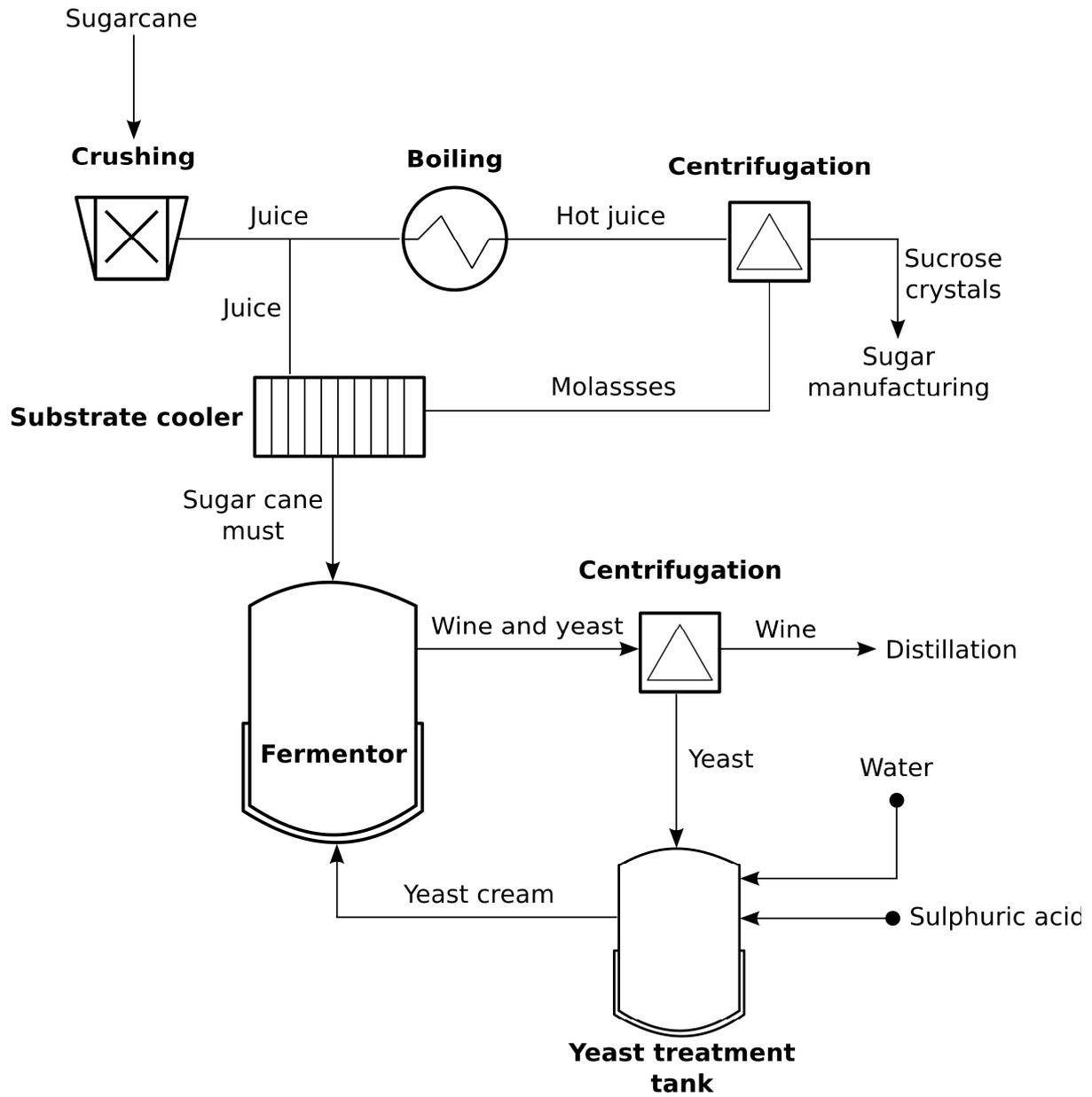


Figure 1 Simplified process flow diagram of the Brazilian ethanol production process (Courtesy of Jens C. F. Nielsen).