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# **Clash of Kingdoms: How Do Bacterial Contaminants Thrive in and Interact with Yeasts during Ethanol Production?**

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## **Abstract**

Brazilian fuel ethanol production from sugarcane is one of the largest industrial biotechnological processes in the world. However, in view of the complex chemical nature of this feedstock, as well as the non-aseptic conditions of the process, various stress conditions are imposed to the fermenting yeast. In this chapter, we deemed to elaborate a brief overview of the ethanol production process, and to dissect the chemical nature of sugarcane-based worts, as well as their physiological effects on the fermenting yeasts. Finally, the interplay between yeast and lactic acid bacteria, the two main players in the ethanol fermentation process, is generally discussed.

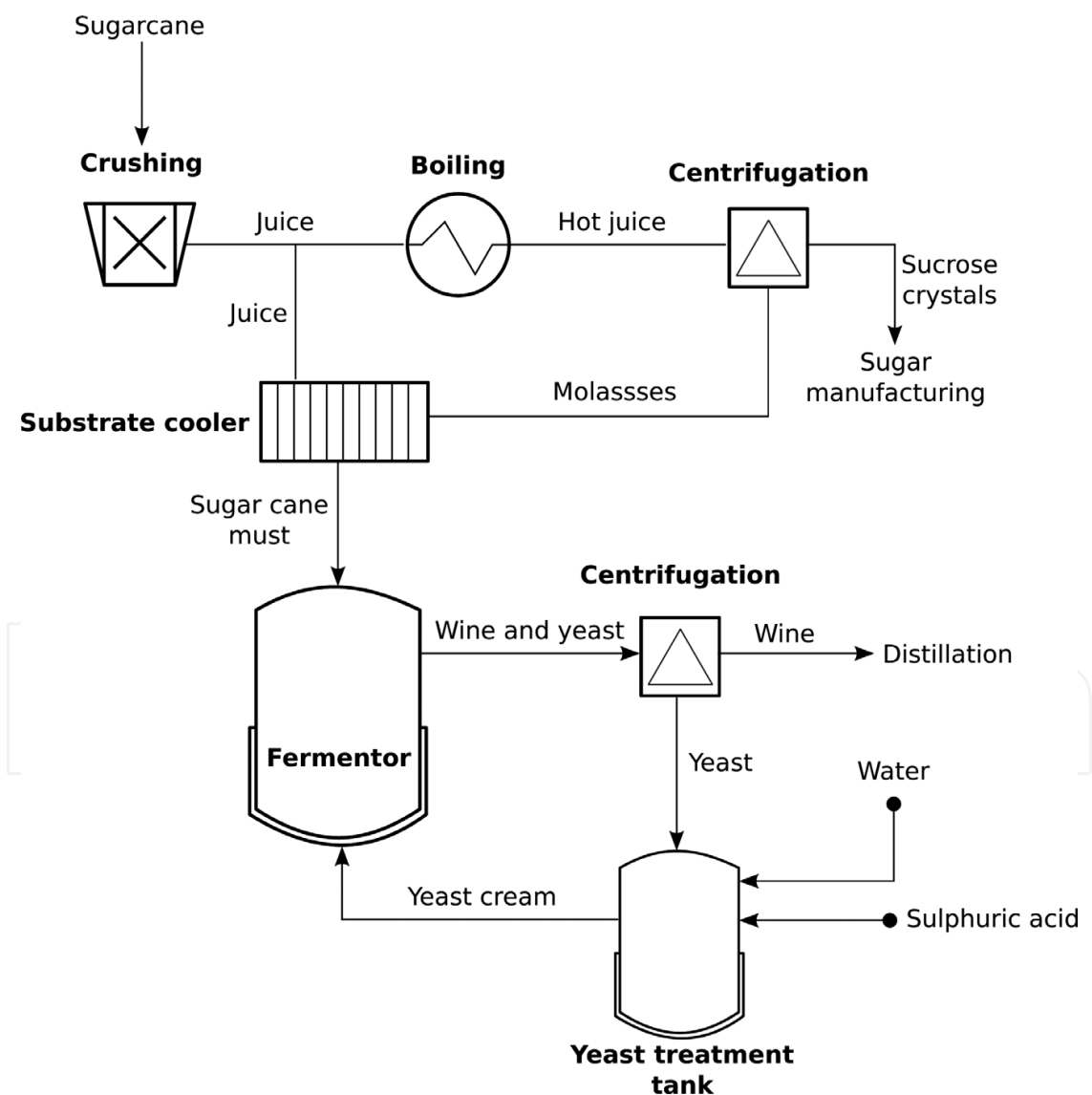
**Keywords:** ethanol, yeast, bacteria, chemical composition, stresses

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## **1. Introduction to ethanol fermentation in Brazil**

Traditionally, ethanol production in Brazil has been coupled to sugar production. Sugarcane is initially pressed to separate the sugar-containing broth (sugarcane juice) from the fibrous solid residue (bagasse). Sucrose crystals are obtained by crystallization of the concentrated broth, and as a result a dark and viscous sucrose-rich residue (molasses) is obtained. Molasses is mixed with either water or sugarcane juice (sugarcane must) in different proportions and used for fermentation, normally in a fed-batch process with cell recycle (for an overview, please refer to [1]).

In short, fermentation is initiated by the addition of the wort (also referred to as must), containing 18–25% (w/v) sugars, to a high-density yeast cell suspension, which represents 1/3 of the vat volume. Due to its large volumes, the feeding takes 4–6 h, and the fermentation is completed within 10 h. At the end of the fermentation, ethanol titres between 8 and 12% (v/v) are obtained, with a final cell density of 10–14% (w/v). Yeasts cells are then separated from the wine by centrifugation, which goes for distillation. The yeast slurry is diluted with equal volume of water and treated with sulfuric acid to reduce bacterial contamination, and reused in a subsequent fermentation cycle (**Figure 1**). This process configuration, using high cell densities and operating with cell recycling, is quite peculiar and allows two fermentation rounds per day during the harvest season that spans for almost 250 days. The reuse of cells reduces the need for yeast propagation, therefore diverting less sugar to yeast growth and saving it to ethanol formation.



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

## 2. Influence of wort composition on fermentation performance

### 2.1. The chemical nature of sugarcane substrates and its by-products

As mentioned above, ethanol can be produced via direct fermentation of sugarcane juice, a mixture of juice and molasses, or molasses diluted in water [2, 3]. After shredding, cane is crushed in a milling tandem, constantly mixed with water, resulting in juices containing ca. 10–15% sucrose content [3]. This process results in two types of juice: the primary – which leaves the first set of miller and is richer in nutrients – and the secondary – coming from the subsequent millers. The primary juice is commonly used for sugar production, whereas the secondary juice can either be used for sugar or ethanol production [3].

Sugarcane juice is passed through clarification (reducing impurities to less than 2%), decantation, and concentration (up to 18–25% total sugars) steps before sugar or ethanol production. These steps help reducing wild yeast and bacterial contaminations during the subsequent fermentation step, allowing for higher ethanol titre and yield [2].

During sugar production, the juice is clarified with lime, and concentrated through repetitive steps of evaporation and centrifugation. The concentrated juice later passes through a crystallization step, which catalyzes the formation of sucrose crystals that are later removed via centrifugation. The remaining sugar is left in the spent, dark and viscous liquid called molasses [4]. Molasses can be further recycled back into the sugar production process, resulting in several other types of molasses (i.e. A, B, C). The higher the number of recycles molasses is subjected to, the poorer its quality as a fermentation substrate [2].

Sugarcane juice and molasses are a complex mixture of carbohydrates, proteins, inorganic salts and organic acids [5], and wort prepared with either juice, molasses mixed with juice, or molasses diluted with water will have different nutrient composition, which will ultimately impact fermentation performance [1]. A comparison between sugarcane juice and molasses composition is shown below (**Table 1**).

Asparagine, glutamine and aspartic acid are the most abundant amino acids in sugarcane-based worts, while the disaccharide sucrose is by far the major sugar. Minor amounts of the monosaccharides, glucose and fructose, and the oligosaccharide, kestose, are also found [5]. Lipids are represented by a mixture of n-alkanes and ethyl and methyl esters of fatty acids (palmitate and oleate are the predominant), as well as of phytosterols (stigmasterol,  $\beta$ -sitosterol, and campesterol) [6, 7].

After fermentation cells are separated from the fermented wort (wine) by centrifugation, and this stream is sent for distillation. The distillation of ethanol generates a considerable amount of a wastewater stream named vinasse (stillage) [8].

Vinasse is currently applied in soil as a fertilizer (fertirrigation), due to its high potassium titre [4, 9]. However, this high organic matter load shows deleterious impacts on soil, water and groundwater [10]. A possible solution to this environmental issue is the anaerobic digestion of vinasse, reducing the organic matter of vinasse for later fertirrigation use, and generating a new stream of revenue (biogas) for the sugarcane ethanol plant [11].

Composition	Sugarcane juice (g/L)	Sugarcane molasses (g/kg)
Total solids	140–190	735–875
Total sugars	105–175	447–587
Sucrose	98–167	157–469
Reducing sugars	6–11	97–399
Nitrogen	0.08–0.3	0.25–1.5
Phosphorous	0.02–0.1	0.3–0.7
Potassium	0.7–1.5	19–54
Calcium	0.1–0.5	6–12
Magnesium	0.1–0.5	4–11

Based on data compiled by [2].

**Table 1.** Composition of sugarcane juice and molasses.

Wort preparation influences final vinasse composition, which will have an impact on the performance of anaerobic digesters, and should be taken in consideration when designing such system. Vinasse can be defined as a mixture of various compounds, including organic acids, mainly fermentation-derived, such as succinic and malic acids, as well as lactic and acetic acids, derived from bacterial contamination. Glycerol is a substantial compound in vinasse, and non-distilled minor amounts of ethanol are also found [11, 12]. A general characterization of vinasse originated from different substrates is depicted in **Table 2**.

**2.2. Substrate related stresses on ethanol fermentation**

Even being successfully used as substrate for ethanol production for decades, sugarcane based-worts present many challenging conditions for the fermenting yeast *S. cerevisiae* [1]. Besides nutrients, industrial worts used in the fermentation process, also carry inhibitors which can be both feedstock- or process-related [13]. During heating steps of juice pre-treatment, some fermentation inhibitors are produced, from sugar degradation (e.g. furfural) and Maillard melanoidins [14].

Furfural, for instance, has been shown to reduce the specific growth rate, the biomass yield on ATP, and both ethanol yield and productivity [15], and is lethal to cells in concentrations above 84 nmol/gDW [16]. During fermentation, furfural is reduced to furfuryl by NADH-dependent alcohol dehydrogenase (ADH) [17, 18], in a NAD<sup>+</sup> regenerative manner, resulting in lower glycerol formation, and higher ethanol titres, when furfural concentration is kept under 29 nmol/l [16].

Tauer et al. (2004) have investigated the effect of Maillard derived products during fermentation of different beverages (i.e. Tequila, Mezcal, whiskey and beer). In their study they observed a reduction in the formation of ethanol of up to 80%. Also, it was observed that the inhibition of these Maillard products is pH dependent, showing little inhibition at pH 4, and increasing at higher pH values.

Wort composition	Parameters (in g/L)									
	COD <sup>a</sup>	BOD <sup>b</sup>	N	P	K	SO <sub>4</sub> <sup>2-</sup>	Ca	Mg	TS <sup>c</sup>	VS <sup>d</sup>
Sugarcane juice	27.7 ± 7.3	14.7 ± 4.9	0.5 ± 0.2	0.11 ± 0.1	1.5 ± 0.8	0.7 ± 1.0	0.4 ± 0.4	0.2 ± 0.1	22.5 ± 1.5	17.9 ± 1.8
Molasses	69 ± 24.7	28.6 ± 13.8	0.9 ± 0.6	0.13 ± 0.21	4.1 ± 2.4	2.3 ± 1.9	1.9 ± 1.4	0.6 ± 0.2	82	60
Mix	37.8 ± 5.8	15.7 ± 4.4	0.5 ± 0.1	0.03 ± 0.04	2.3 ± 1.1	0.9 ± 0.5	1.4 ± 1.1	0.3 ± 0.04	105 ± 52.5	8.4 ± 4.7
Based on data compiled by [11].										
<sup>a</sup> Chemical oxygen demand.										
<sup>b</sup> Biochemical oxygen demand.										
<sup>c</sup> Total solids.										
<sup>d</sup> Volatile solids.										

**Table 2.** Vinasse composition from different sugarcane-based worts.

Sugarcane juice also presents potentially toxic metal ions on its composition. Walford (1996) presented a compilation of mineral values from sugarcane juices (**Table 3**).

Aluminum shows high toxicity under acidic conditions (being present in  $\text{Al}^{3+}$  form) and is particularly deleterious to yeast cells, reducing cell viability, trehalose content, and ethanol yield [1]. Its deleterious effects can be alleviated by magnesium concentration in the broth, or completely abolished by mixing sugarcane juice with molasses. This might be related to some chelating property of molasses [2].

Other metal ions also play an important role in yeast fermentation inhibition. Sugarcane molasses showed inhibitory effect towards invertase activity of a laboratory *S. cerevisiae* strain. This inhibition could be further replicated in laboratory media when copper ions ( $\text{CuCl}_2$ ) where added in the media at a concentration of 0.04 M [19].

Another important factor is the osmotic stress that is caused by elevated concentration of salts. Cations such as  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^+$ ,  $\text{Na}^+$ , and anions, like  $\text{Cl}^-$  and  $\text{SO}_4^{2-}$ , can all have severe effects on yeast growth and ethanol production [20].

Sugarcane juice and molasses have a complex composition of organic acids. Even though they do not respond to a major fraction of the composition of these raw materials, they are responsible for their pH values (ca. 5–6) and their buffering capacity [5]. The composition, and concentration, of such acids depend on several factors, such as the maturity stage and variety of the plant, weather, soil and health state [21]. The most common organic acids found in sugarcane are trans-aconitic (5000–8000 ppm/Brix), malic (1200–1800 ppm/Brix) and citric

Component	Concentration (% on total solids)
K <sub>2</sub> O	0.77–1.31
Na <sub>2</sub> O	0.01–0.04
CaO	0.24–0.48
MgO	0.1–0.39
Al <sub>2</sub> O <sub>3</sub>	0.005–0.17
Fe <sub>2</sub> O <sub>3</sub>	0.006–0.04
CuO	0.002–0.003
ZnO	0.003–0.012
MnO	0.007
CoO	0.00007
SO <sub>3</sub>	0.017–0.52
P <sub>2</sub> O <sub>5</sub>	0.14–0.4
Cl	0.16–0.27
SiO <sub>2</sub>	0.016–0.101

Based on data compiled by [5].

**Table 3.** Inorganic composition of sugarcane juice.



(900–1800 ppm/Brix) acids. Other acids found in lower concentrations are succinic, oxalic, tartaric and glycolic acids [5]. During fermentation, lactic and acetic acids are also formed via contaminating bacteria.

Undissociated organic acids are lipid permeable and thus enter yeast cells. Due to the higher intracellular pH as compared to the environment, dissociation into the corresponding anion, leads to intracellular acidification and ATP expenditure, a mechanism known as weak acid uncoupling [22], which is described in more details in the following sections. Moreover, the anion accumulated inside the cells may reach toxic concentrations that impair essential metabolic functions.

### 3. The interplay between yeast and bacteria in the fermentation process

Fuel ethanol fermentation performed in Brazil is one of the largest industrial biotechnological processes in the world, with the most favorable energy balance as compared to other similar processes for ethanol production [13, 23]. However, in view of the nature of the process and the large volumes processed, aseptic conditions are never achieved. Therefore, bacterial contamination is a concurrent problem in industrial fermentations.

This is regarded as a major drawback that deviates sugars away from ethanol formation and lead to detrimental effects upon yeast fermentative performance, such as reduced ethanol yield, yeast cell flocculation, and low yeast viability [24–27].

#### 3.1. Homo- and heterofermentative lactic acid bacteria

Bacterial contaminants found present in the fermentation step of ethanol production comprise mainly lactic acid bacteria (LAB) [28], probably because of their higher tolerance towards acidic pH and ethanol titres when compared to other microorganisms [29, 30]. Studies that investigated the identity of these contaminants during yeast fermentation in Brazilian ethanol plants found that *Lactobacillus* was the most abundant genus [28].

Contaminating lactic acid bacteria are traditionally classified in two major metabolic subgroups according to the pathway used to metabolize hexose sugars: homo- and heterofermentative [29]. In general, bacteria isolates from industrial fermented sugarcane substrates have shown to include both types [31].

Homofermentative bacteria catabolize hexoses via the so-called Embden-Meyerhof-Parnas (EMP) pathway, in which 1 mol of hexose results in the formation of 2 mol of lactic acid and 2 mol of ATP. In comparison, in heterofermentative bacteria another pathway is active, 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway [29], and hexoses are converted to equimolar amounts of lactic acid, ethanol or acetate, and carbon dioxide, yielding 1 mol of ATP per mol of hexose fermented [32, 33]. With the conversion of acetyl phosphate to acetate instead of ethanol, an additional ATP can be produced. Then, regeneration of surplus NAD<sup>+</sup> must be achieved by an alternative electron acceptor. Under aerobic conditions, oxygen may serve as the electron acceptor [34], but under anaerobic or even oxygen-limited conditions, fructose is reduced to mannitol, serving as an electron sink [35]. There is a third classification

group that, differently from the homofermentative strains that cannot metabolize pentose sugars, they can ferment these sugars using an inducible phosphoketolase pathway, producing lactate and acetate [36].

### 3.2. LAB effects on the fermenting yeast

Contaminating bacteria found in ethanol fermentations are often fastidious organisms which compete for nutrients against the fermenting yeast, negatively impacting its fermentation performance [25]. These nutrients are often several growth factors like nucleotides, amino acids and vitamins [25]. Due to the fast-growing nature of these bacterial strains, wort can become rapidly depleted for such nutrients [37]. This nutritional deficiency might result in lower ethanol titres, lower yeast crop viability and budding, longer fermentation periods and higher contents of residual sugars, a phenomenon called stuck or sluggish fermentation [27].

Concomitantly to nutritional competition, these contaminants also deviate carbon to their catabolic pathways and produces organic acids and polyalcohols [27]. These weak organic acids also have a deleterious effect on yeast metabolism. In low pH conditions, usually found in fermentations (i.e. 6.5–4.5) they are found mostly in their protonated form, due to their high pK<sub>a</sub> values (3.86 for lactic acid; 4.75 for acetic acid) [27]. In this form, these uncharged molecules can permeate the plasma membrane and, when encounters the cytosolic pH (7.0), rapidly dissociates releasing its proton and acidifies the intracellular pH (pH<sub>i</sub>) [38].

This acidification of the pH<sub>i</sub> affects many cellular processes, by influencing the ionization states of acidic and basic side chains of amino acids in important enzymes, changing their tertiary structure and activity [39]. Among the several vital functions that are inhibited by pH<sub>i</sub> acidification is the glycolysis [40], thus inhibiting the cell's ability to recover ATP.

*Saccharomyces* responds, partially, to this acidification via the plasma membrane H<sup>+</sup>-ATPase pump, Pma1p. It pumps out H<sup>+</sup> using ATP hydrolysis, at a 1:1 ratio [41]. This response mechanism comes with a cost: almost 20% of all ATP formed during normal conditions is drained by this process<sup>40</sup>; at starvation conditions, when exposed to weak acid stress, the amount of consumed ATP can go as high as 60% [42].

Other responses are also triggered, when yeast cells are exposed to weak acid stress. The plasma membrane ATP binding cassette (ABC) transporter Pdr12p is induced when cells face this stressful condition [43], and it is believed to play an important role on yeast cells adaptation to grow in the presence of weak organic acids by pumping out H<sup>+</sup> ions, under the expense of either ATP or proton gradient [42].

When protons and anions are pumped out of cells, they re-associate. Once protonated, these molecules can permeate again to the interior of yeast cells, forcing them to pump out these ions repeatedly. This process is defined as a futile cycle<sup>36</sup> and is a major energetic drain in industrial processes. These energetic drains increase cells maintenance coefficient (m) and reduce their fitness, inhibiting their growth and reducing their viability [44].

Despite these universal cellular responses, yeast might be more or less susceptible to a given contaminant, depending on its metabolism and also on the physiological context these organisms are exposed to. Growth conditions such as temperature, pH, sugar content, nutrients



availability and biological factors like yeast strain, population density and bacterial metabolism should all be considered when analyzing the impacts of contaminant species in industrial fermentations.

For instance, previous studies have demonstrated that, under certain fermentation conditions, competition towards nutrients is the most probable cause for yeast cells inhibition. *S. cerevisiae* in steady-state pure cultures withstood high concentrations of added lactic acid, without losing much of cell viability nor ethanol production/productivity, when compared to co-cultivated *S. cerevisiae* and the facultative heterofermentative *L. paracasei* [26]. This shows that organic acid production by bacterial metabolism might not be, in some circumstances, directly involved in yeast cells stress.

### 3.3. Co-cultivation studies

Co-cultivations experiments, in the context of ethanol production, are rather scarce in the literature. Very recently, a quite interesting investigation was performed using two common contaminant microorganisms in industrial ethanol plants, the heterofermentative bacterium *L. fermentum*, and the contaminating yeast, *Dekkera bruxellensis* [45]. The authors found that during co-cultivation experiments with *S. cerevisiae* strain PE-2 in the presence of both contaminants, the growth of the contaminating yeast was stimulated by the presence of the bacterium. This condition resulted in a more pronounced effect on the fermentation parameters than the effects observed in binary combinations of the fermenting yeast (PE-2) with each contaminant.

Wild contaminants of *S. cerevisiae* were also evaluated in co-cultivations experiments [46]. They were found to be more detrimental to fermentative performance (resulting in lower ethanol production and higher residual sugars) when compared to co-cultivations with a heterofermentative bacterium.

Bacteria showing different metabolic pathways may also impact differently yeast physiology and ethanol fermentation, on a context-dependent manner. Homofermentative bacteria (i.e. *L. plantarum*) was more inhibitory to yeast cells than heterofermentative bacteria (i.e. *L. fermentum*), when these strains were co-cultured with an industrial *S. cerevisiae* strain (CAT-1), under laboratory conditions (i.e. in equal cell concentrations). When the context of sugarcane ethanol fermentations is put in perspective (i.e. short fermentations catalyzed by high cell densities) *L. fermentum* were more deleterious, outcompeting yeast cells in the fermentation process. In these conditions, the fastidious metabolism of *L. fermentum*, and its faster uptake of fructose – a sugar typically not consumed as fast as glucose by *S. cerevisiae* – may have given *L. fermentum* a competitive advantage, when compared to *L. plantarum*, which had to compete with *S. cerevisiae* for the available glucose [27]. Therefore, it was concluded that under conditions similar to those used in the industrial production of fuel ethanol, heterofermentative strains have a more detrimental effect over yeast performance, in terms of ethanol yield and yeast viability.

### 3.4. Monitoring bacterial contamination

Mannitol is a suitable indicator of sugarcane deterioration and bacterial contamination during industrial fuel ethanol fermentation [47, 48]. Glucose and fructose normally present a 1:1 ratio,

since sucrose is the prevalent sugar in this feedstock. Therefore, mannitol titres can be used to predict sucrose losses due to bacterial contamination as well as dextran synthesis, which results in problems such as viscosity, evaporation, crystallization and, to a lesser extent, poor filterability in sugarcane factories [49].

Another interesting indicator of bacterial contamination is lactic acid. This organic acid is considered by many a suitable indicator of bacterial contamination during industrial ethanol fermentation. However, because D- and L-lactic acids is formed by industrial lactobacilli isolates [50], as a result of varying proportions of racemases [51, 52], conflicting results are expected depending on the technique employed to quantify this by-product. This is because most commercial enzymatic kits usually employed in the routine analysis of lactic acid in ethanol plants, normally detect only the L-form.

#### 4. Concluding remarks

Industrial yeast cells are exposed to several stresses during sugarcane ethanol production. These stresses might be related to media composition, but also to the microbial community composition found in industrial fermenters. The understanding of the physiological responses of yeasts towards these stresses is paramount for improving current and future *S. cerevisiae* catalysed sugarcane-based bioprocesses.

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#### Conflict of interest

The authors declare that they have no conflict of interest.

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