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Ethanol Production in Brazil: The Industrial Process and Its Impact on Yeast Fermentation

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1. Introduction

Ethanol is the most consumed biofuel in the world. Brazil is the country that first introduced this renewable fuel in its energy matrix. A large industry arose from this pioneering initiative, and nowadays detains the most economically feasible process for bioethanol production. For decades, Brazil was the main producer but was recently surpassed by the United States. Nevertheless, Brazil is currently the greatest ethanol exporter. Many factors contributed for the efficiency of this industry, as: feedstock, fermentation and process improvements. In this chapter, general aspects of the Brazilian ethanol fermentation process will be exhibited, many improvements will be highlighted, and the impacts of the peculiarities of this process on yeast fermentation will be discussed.

2. A brief history of the Brazilian ethanol industry

Ethanol is the main biofuel used for transportation and Brazil is the second largest bioethanol producer and the greatest exporter. The United States (the greatest producer) and Brazil are responsible for 70 % of the world ethanol production (RFA, 2011). The sugar and ethanol industry in Brazil make up 2.3 % of the Domestic Gross Product, generating 4.5 million jobs. Additionally, fuel ethanol represents almost 50 % of the total fuel volume consumed by cars (and light vehicles).

Sugar cane was introduced in Brazil by the Portuguese colonizers at the beginning of the 16th century. The first mills for cane sugar production (consumed in Europe) were established around the year 1530 (Amorim and Leão, 2005). Today, sugar cane is an important crop in Brazil occupying ca. 8 million hectares with a production of more than 600 millions tons per year, making the country the largest sugar cane producer worldwide.

The Brazilian knowledge in ethanol production from sugarcane began to be developed in colonial period, when farmers used to produce the Brazilian sugarcane distilled spirit, the "cachaça" (Basso and Rosa, 2010). Then, since the beginning of the 20th century, Brazil has been

using ethanol for energetic purposes. In 1905, the first tests of using ethanol as fuel for vehicles engines were performed. These tests provoked official attention and resulted in a law published in 1931, which determined that ethanol should be mixed to the gasoline at a rate of 5 % (v/v). As any new technology, ethanol needed economic and political investments to keep its viability in national market and also, to fight against a major competitor: petrol.

Winds had changed in early 1970's, when oil crisis tripled oil imports costs to Brazil in 1973, due to the Arab oil embargo. In addition, world sugar prices, which had been climbing upward since the mid-60's, declined sharply in 1974 (Sandalow, 2006). During these drastic changes in global fuel market, the country launched the Brazilian National Alcohol Program (PROALCOOL) in 1975, aiming at large scale ethanol generation by local distilleries and engines adaptation to consume the E20 mix (20 % and 80 % ethanol, gasoline, respectively) or even pure anhydrous ethanol (Amorim and Leão, 2005).

To improve ethanol competitiveness, government offered low-interest loans for construction of new refineries and gasoline prices were set to give ethanol a competitive advantage. Brazilian state-owned oil company (Petrobras), began making investments for distribution of ethanol throughout the country. After this intervention, ethanol production boomed more than 500 % (Sandalow, 2006).

In the 1980´s, the major car companies accorded to install assembly lines for 100 % ethanol cars. This resulted in a high demand, which reached the peak at the half of the decade, when ethanol supply as fuel for vehicles was the half of the total fuel consumed in Brazil.

Oil prices dropped sharply in 1985-86. In parallel, a bad economic period (illustrated by a high inflation) led the government to cut subsides to the ethanol industry, leading to the fuel shortage in the market. However, during the 1990´s the Brazilian economy has lifted. In addition, the energy world market, especially the oil price and Asia crisis, were favourable for the ethanol production (Moreira, 2000).

At the first decade of 2000, flex-fuel engines were exclusively designed locally to Brazilian market. These engines let the consumers to choose between ethanol and gasoline, depending on its price on the market. Indirectly, this also facilitated the ethanol market regulation. Currently most of the produced ethanol is consumed internally. Of all automobiles used in Brazil, eight out of ten are flex fuel vehicles – more than in any other country in the world (Pilgrim, 2009).

3. The industrial process

3.1 Feedstock

Technically, ethanol can be produced from a wide variety of renewable feedstock, which can be roughly classified into three main groups: (1) those containing considerable amounts of readily fermentable sugars (sugar cane, sugar beets, sweet sorghum), (2) starches and fructosans (corn, potatoes, rice, wheat, agave) and (3) cellulosics (stover, grasses, corn cobs, wood, sugar cane bagasse). Sugar cane, beet and sweet sorghum provide the simple sugars, as sucrose, glucose and fructose that can be readily fermented by yeasts (Amorim et al. 2009). This differs from ethanol production processes based on starchy or lignocellulosic feedstock, where prior hydrolysis of polysaccharides is necessary with increased ethanol production costs (Dien and Bothast, 2009).

The feedstock has a great impact on ethanol production costs, which is also influenced by the region and by the processing. The production costs are affected by improvement in technologies and vary during time-to-time, making a realistic comparison a difficult task (Figure 1).

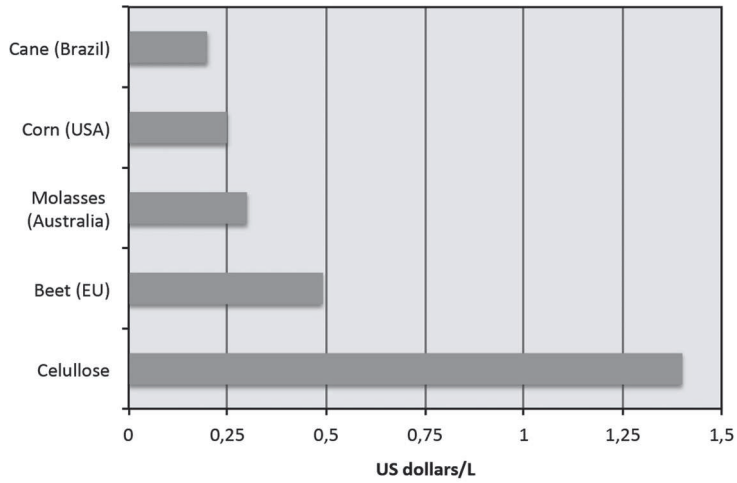

Fig. 1. Gross estimation of ethanol production costs in US dollars/litres from different feedstock and regions, from several sources available in 2007 (Burnquist, 2007).

As a C-4 photosynthetic species, sugar cane presents very high biomass productivity, amounting to 80-120 ton/ha.year with an industrial ethanol production of 8,000 litres/ha, higher when compared to 3,000 litres/ha from maize. Nitrogen-fixing endophytic bacteria (mainly *Acetobacter diazotrophicus*, *Azospirillum* spp. and *Herbaspirillum* spp., *Gluconacetobacter diazotropicus*) have been discovered in both sugar cane and in maize. It has been suggested that at least 60 % of the plant's nitrogen requirement, is supplied endogenously when sugar cane is grown in low fertility soil (James and Olivares, 1997; Boddley, 1995; Wheals et al., 1999; Bertalan et al., 2009). As nitrogen fertilizers are quite expensive and require huge amounts of fossil energy for its production, sugar cane presents economic and environmental benefits when compared to other crops. Its drought tolerance also contributes for some agricultural advantages.

Sugarcane contains 12-17 % total sugars (90 % sucrose and 10 % glucose plus fructose) on a wet-weight basis with 68-72 % moisture. The sugar average extraction efficiency by crushing (or diffusing) is approximately 95-97 % (Wheals et al., 1999). Bagasse plays an important role in the energy balance of sugar cane ethanol. It comprises 20 to 30 % (with 50 % moisture) of sugar cane on a wet-weight basis and it is used for steam generation for milling, heating, distilling and more recently for electricity co-generation, making an ethanol plant not only self-sufficient but an energy exporter.

After the beginning of the new millennium, several Brazilian distilleries began to invest in the acquisition of more efficient high-pressure boilers (steam operation pressure up to 80 bar), yielding 120 kWh/ton of bagasse. Electricity co-generation pulled up the already positive energy balance. According to Coelho et al. (2006), energy balance in ethanol industry can be higher than 10:1 (output:input). Leite et al. (2005) estimated 8:1. The main energy spent in sugarcane ethanol is due to agricultural features, mainly to the use of fertilizers and transportation. Regarding maize, Shapouri et al. (2008) concluded that energy balance for corn-based ethanol distilleries was 2.3:1 or even 2.8:1. These values are much higher than the balance shown by Pimentel (2003) who proposed that there were no energy gains to produce ethanol from corn, as the energy balance was 1:1. In addition, energy

balances for ethanol generated from lignocellulosics and sugar beet were 5.6:1 and 2:1, respectively (Elsayed et al, 2003).
Furthermore, as transport of loads in Brazil is predominantly done by road using low capacity trucks, this item pull up the energy spent. Improvements in this area would become ethanol from sugarcane even more feasible. It is also expected that the very high gravity (VHG) fermentation will make such figures even better, due to energy savings in distilling and mainly for vinasse transportation to the field, since this by-product will be generated in a more concentrated form and consequently in a lower volume. Therefore, energy balance in ethanol production by sugarcane is highly positive and much higher than ethanol generated from other sources.

3.2 Substrate for fermentation

Traditionally ethanol production was coupled with sugar industries in Brazil (Basso and Rosa, 2010). The cane sugar is pressed (some plants use diffusion), resulting in sugar cane juice and a solid fibrous residue, the cane bagasse. After clarification the juice is concentrated by evaporation till sucrose crystallization. The sucrose crystals are collected by centrifugation, generating a sucrose saturated viscous phase, called cane molasses with 45 to 60 % sucrose and 5 to 20 % glucose plus fructose. Initially, the production of ethanol was established as a way to process the resulting molasses from the sugar industry, but due to the increasing importance of ethanol in the 80's, many mills began as autonomous ethanol plants. Over time, additional investment enabled mills to direct the cane sugar to either row sugar or ethanol, and today only a few facilities were dedicated to produce exclusively ethanol and no sugar. Thereafter, the Brazilian industrial process for fuel ethanol production started to use sugar cane juice and molasses as substrates, mixed in different proportions. Indeed, some distilleries use only juice, while others only molasses, but the mixture is considered to be a better substrate, since the juice has some nutritional deficiencies, whereas molasses has inhibitory compounds for yeast fermentation.
The mineral composition of sugar cane substrates vary widely, depending on the molasses proportion used to formulate the media, sugar cane variety and maturity, soil, climate, and processing of cane juice (Table 1).

Nutrient	**Range concentration* (mg/L)**	**Optimum level* (mg/L)**
Nitrogen (NH_4^+ and R-NH_2)	70-350	100-300
Phosphorus	20-200	50-250
Potassium	300-12,000	700-1,300
Magnesium	80-3,900	100-200
Sulphur	80-3,900	as low as possible
Calcium	150-2,000	as low as possible
Zinc	0.45-9	1-5
Copper	0,20-8	1-5
Manganese	2-8	1-5
Aluminium**	2-500	<10 (in juice substrate)

Table 1. Mineral composition of sugar cane based substrates and the recommended levels for yeast fermentation (Amorim and Leão, 2005). *Concentration of the element; **Not a nutrient, but a toxicant.

3.3 The fermentation

The process operates in fed-batch (75 % of the distilleries), also mentioned as Melle-Boinot process, or in continuous mode, both utilizing yeast cell recycling. In both process, after the end of fermentation, yeast cells are collected by centrifuging and re-used in a next fermentation cycle. Up to 90-95 % of the yeast cells are recycled, resulting in high cell densities inside the fermentor [10 to 14 % (wet weight basis/v)]. Cell reuse reduces the need for intensive yeast propagation, and less sugar is deviated for biomass formation. It is estimated that yeast biomass increases 5 to 10 % (in relation to initial biomass) during a fermentation cycle, which is enough to replace the yeast cells lost during the centrifugation step. This high yeast biomass inside the fermentor is responsible for a very short fermentation time of 6 to 10 h, when compared to 40 to 50 h in corn fermentation process. Normally, temperature is kept around 32 to 35 °C, yet due to the short fermentation time, cooling is not always efficient enough in removing heat, and temperature may reach up to 40 °C, especially in the summer season (Lima et al., 2001; Laluce, 1991; Wheals et al., 1999; Lima et al., 2001; Amorim et al, 2004; Andrietta et al., 2002).

Generally, fermentation starts by adding cane must (prepared by mixing cane juice and molasses at any proportion), which contains 18 to 22 % (w/w) total reducing sugars (TRS), to a yeast cell suspension. This yeast suspension (with ca 30 % of yeast cell, on wet basis) represents 25 to 30 % of the total volume of fermentation, and is performed in tanks of 300 to 3,000 m^3. This large inoculum is normally prepared by mixing 2 to 12 ton of baker's yeast with 10 to 300 kg of selected strains in active dry yeast form. The feeding time normally lasts for 4 to 6 h and fermentation is finished within 6 to 10 h, resulting in ethanol titres of 8 to 12 % (v/v). When fermentation ceases, yeast cells are separated by centrifugation, resulting in a concentrated yeast cell suspension (the yeast "cream") with 60 to 70 % (wet weight basis/v) of cells. The yeast cream is diluted with water (1:1), and treated with sulphuric acid (pH 1.8-2.5) for 2 h, in order to reduce bacterial contamination and to be re-used as starter for a next fermentation cycle. This recycling trait makes the Brazilian process quite peculiar in that yeast cells are reused at least twice a day over the production season of 200-250 days. Tank stirring is also desirable at a low power, only to avoid cells compacting at the bottom of the tank and to keep a higher contact surface with substrate.

After centrifuging, the "beer" or "wine" is driven to distillation for ethanol recovery, normally using distillation tray technology. After distilling a liquid stream called stillage or "vinasse" is produced at the ratio of 10-15 litters per litter of produced ethanol and is delivered into the cane fields for use as irrigation water and fertiliser (adding to soil potassium, calcium, magnesium, others micronutrients and some organic matter).

When compared to the continuous mode, Melle-Boinot process (Figure 1) presented higher yields and productivity, lower levels of contamination and it was easier to clean (Zhang, 2009), but according to Andrietta et al. (2007) beneficial traits of a continuous version are masked by the improper engineering conception of low-cost adaptations of batch to continuous plants.

4. The impact of industrial conditions on yeast physiology and population dynamics

4.1 Industrial stresses

As seen before, industrial yeast strains encounter several simultaneous or sequential stress conditions imposed by the process itself, such as: high ethanol titters, high osmotic pressure, low pH, high temperature, and many others, all of them intensified by the practice of cell recycling.

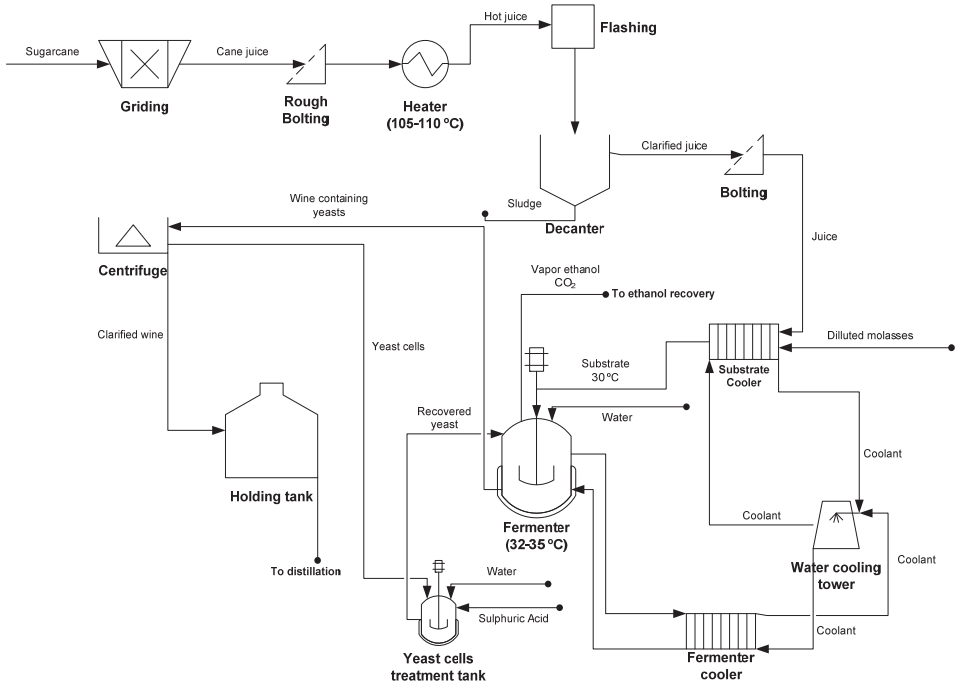

Fig. 1. Scheme of the fed-batch process with cell recycling (Melle-Boinot).

4.1.1 Osmotic stress

Osmotic stress is frequently mentioned in different industrial processes. In sugar cane-based fuel ethanol fermentation, yeasts are often exposed to high sugar concentrations, since a final ethanol content of 8-12 % (v/v) is very common to be achieved. In theory, the sugar concentration needed to result in ethanol titers of 12 % will be around 20 % (w/v) of TRS, considering an ethanol yield of 90 %. However, yeasts never face such sugar concentrations during industrial fermentations. This is because during the fed-batch process, with 4-6 h of feeding time, total sugar concentrations hardly exceeds 5-7 % (w/w). Indeed, in some occasions, sugar consumption rate by yeast cells equals the sugar-feeding rate, and very low levels of sugars [< 2 % (w/v)] are attained during fermentation. Therefore, one can deduce that the osmotic stress imposed by sugars can be practically neglected, especially in the case of fuel ethanol production performed in fed-batch mode.

On the other hand, the osmotic stress caused by salts, which are present in great amounts in sugar cane molasses, is a matter of concern. High levels of potassium, calcium and magnesium found in this substrate, all exceed by far the requirements for yeast nutrition. Average levels of potassium (4,000 mg/L) are high enough to induce stress responses in fermenting yeasts, increasing glycerol formation, reducing yeast storage carbohydrates and lowering ethanol yield (Alves, 2000).

It is often observed an increased formation of glycerol during osmotic stress conditions (Brumm & Hebeda, 1988; Myers et al, 1997), as well as during other stress situations, such as bacterial contamination (Alves, 1994; Gomes, 2009). This suggests that increased formation

of this metabolite might be an indicative of general stress conditions during industrial fermentations. Glycerol is the major by-product during fermentation, mainly formed as a result of re-oxidation reactions to consume the excess NADH formed during biosynthesis under anaerobic conditions (van Dijken e Scheffers, 1986). As a result, during industrial fermentations 5 to 8 % of the sugars consumed by yeast cells are diverted into glycerol (Oura, 1977). Lowering the amount of this polyol is considered a promising route to increase ethanol yield. This could be achieved by adjusting the feeding rate during the fed-batch process, or by selecting strains that produce less glycerol. Both approaches are considered promising routes to increase ethanol yield, and these have been observed both in laboratory conditions as well as in the industrial practice.

4.1.2 Ethanol stress

In view of the high titers of ethanol towards the end of each fermentation cycle [8-12 % (v/v)], this alcohol is one of the major stress factors that act upon yeast. The inhibitory role of ethanol upon *S. cerevisiae* is not fully understood until now. Even so, the main target of ethanol is considered to be the cytoplasmatic membrane of yeast cells (Thomas et al., 1978; Alexandre et al., 2001). The membrane fluidity, which is related to its lipid composition, is profoundly altered in the presence of ethanol, and, as a result, membrane permeability to some ions (especially ions H^+) is significantly affected. As ions enters the cell, there is a dissipation of the electro-chemical gradient across the membrane, which in turns affects formation and maintenance of the proton driving force with subsequent decrease in intracellular pH. Apart from affecting yeast membrane composition, there are several other effects of ethanol upon yeast physiology during fermentation, including growth inhibition, and enzymatic inactivation, which leads to a decreased cell viability.

Cell re-use (yeast recycling) imposes a harsh condition towards industrial strains. Yeast cells must keep high viability at the end of each fermentation cycle in order to be able to cope with the following one. This is why a given strain can perform well in one fermentation cycle with a final ethanol content of 18 % (v/v), but can not be recycled for subsequent fermentation cycles. Indeed, if yeast physiological condition, namely the vitality of the cells at the end of the fermentation, is not a matter of concern, higher ethanol contents could easily be achieved. This is observed in some corn and cereal-based fermentation processes, where 17 to 23 % (v/v) of ethanol titers are obtained when wheat and formulated mashes are used (Jones et al., 1994; Bayrock and Ingledew, 2001).

Despite these problems, high ethanol fermentations are desirable in order to reduce water consumption and energy costs during the distillation step. It is also expected that this fermentation condition will favour the energy balance of the produced ethanol and improve the sustainability of the industrial process. However, in most distilleries the final ethanol content is limited by the ethanol tolerance of the fermenting strain. Additionally, ethanol stress can be intensified by high temperature and acidity, and all these stressing factors are imposed simultaneously or sequentially towards yeasts in industrial fermentations (Dorta et al., 2006).

A great research effort is now being conducted to search for ethanol tolerant yeast strains, in order to be used in high ethanol fermentations, specifically for the case of cell recycling.

The genetic basis for ethanol tolerance is polygenic and complex. More than 250 genes seem to be involved with this trait (Alexandre et al., 2001; Hu et al., 2007). The majority of these genes are related to energy metabolism, lipid synthesis, ionic homeostasis, trehalose synthesis, etc. This means that yeast improvement for ethanol tolerance through a rational approach is a difficult task, and a more "blind" approach (such as genome shuffling,

mutagenesis, directed evolution) could be helpful in the search for such strains (Stephanopoulos, 2002; Çakar et al., 2005; Giudici et al., 2005).

4.1.3 Acid stress

Although it is well known that yeasts generally can tolerate low pH, the sulphuric acid treatment of "yeast cream" (pH 1.8 to 2.5 during a couple of hours), in order to reduce bacterial contamination, causes physiological disturbances in yeast cells. This is observed by minerals (N, P, K, Mg) leakage and a decreasing level of yeast cellular trehalose in parallel with cell viability drop (Ferreira et al. 1999). Yeast strains that tolerate the stressing conditions of industrial fermentations normally present higher trehalose levels (Basso et al., 2008). Undoubtedly, undissociated organic weak acids present in the substrate or produced by contaminating bacteria can lower intracellular pH followed by ion translocation across the yeast plasma membrane. These events are intensified by the low pH of the fermentation medium. Residual levels of sulphite (SO_2), used for cane juice clarification, can be found particularly in molasses substrates. Although considered a toxicant towards yeast at a level of 200 $mg.L^{-1}$, sulphite can be considered beneficial to the fermentation, once it can reduce bacterial contamination (Alves, 1994).

4.1.4 Other stresses

The presence of toxic levels of aluminium in cane based industrial substrates is also 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 (Al^{3+}), leading to serious problems during fermentation. It negatively affects yeast viability, cellular trehalose levels, fermentation rate, and ethanol yield. The toxic effects of aluminium can be partially alleviated by magnesium ions and completely abolished in a molasses rich medium, suggesting the presence of chelating compounds in this substrate (Basso et al., 2004). Industrial yeast strains differ greatly regarding aluminium tolerance, in terms of cell viability, ethanol yield and aluminium cell accumulation. *S. cerevisiae* CAT-1 is less sensitive in comparison to PE-2 and commercial baker's yeast. In industrial practice, high levels of aluminium are associated with low cell viability and reduced ethanol yield.

Very low levels of cadmium were found in sugar cane from an area fertilized with treated municipal sewage sludge (Silva et al. 2010). Even so, the cell recycling and the yeast capacity of cadmium accumulation led to a toxic level of this metal to yeast fermentation. Yeast cells presented low viability, reduced sugar uptake, reduced trehalose levels and low ethanol yield. Vinasse (the effluent generated after ethanol distillation), when used as a substrate supplement, showed to be efficient in reducing the toxic action of aluminium (Oliveira et al., 2009) and cadmium (Mariano-da-Silva, 2001; Mariano-da-Silva and Basso, 2004) towards yeast. It is believed that chelating compounds present in vinasse were responsible to mitigate the toxic effects of heavy metals.

Several attempts were done in order to select thermo-tolerant strains for industrial fermentation (Laluce, 1991). On the other hand, high temperature is known to intensify ethanol and acidic stresses upon yeasts. As bacterial contamination is strongly stimulated by temperature above 32 °C, it is argued whether higher temperatures would be feasible. Nowadays, due to the advantages of the use of very high gravity (VHG) fermentations, lower temperature (27 °C) is appointed as a way to reduce the alcohol toxicity. All these suggestions are still to be evaluated in industrial scale.

Other stressors, such as herbicides used in sugar cane fields, phenols found in sugar cane juice, excessive amounts of iron in molasses, and others, are likely to be affecting yeasts during industrial fermentations, but their effects are still to be demonstrated. Additionally, the drastic changes in physiological conditions during fermentation as the rate of feeding, acid treatment and the frequent stoppage, all contribute to severely affect yeast fermentative performance.

4.1.5 Bacterial contamination

In view of the nature of the industrial process and its large volumes of processed substrates, aseptic conditions are very difficult to be achieved, and fermentation normally operates with bacterial contamination. This is often regarded as a major drawback during industrial ethanol fermentation. Besides deviating feedstock sugars from ethanol formation, there are also detrimental effects of bacterial metabolites (such as lactic and acetic acids) upon yeast fermentative performance, resulting in reduced ethanol yield, yeast cell flocculation, foam formation and low yeast viability (Yokoya et al., 1997; Narendranath et al., 1997; Bayrock & Ingledew, 2004; Eggleston et al. 2007). Induced yeast flocculation impairs the centrifuge efficiency and reduces the contact surface between yeast and the medium. Excessive foam formation increases costs due to the use of more antifoam chemicals in order to reduce headspace. The antibiotics, used to decrease contamination, increase costs and their residual levels make dried yeast (a by-product of ethanol industry) improper for commercialization (for human consumption or animal feed).

Most of the bacterial contaminants during the fermentative step of ethanol production are lactic acid bacteria (LAB), probably because of their higher ability to cope with the low pH values and high ethanol concentrations compared to others microorganisms (Kandler, 1983; Skinner and Leathers, 2004).

In a survey that investigated the identity of these contaminants during industrial fermentation in ethanol plants (distilleries) located in Brazil, Gallo et al. (1990) found that *Lactobacillus* was the most abundant isolated genera. Recently, Lucena et al. (2010) showed that LAB are the most common contaminants in distilleries located at the Northeast region of Brazil.

LAB are traditionally classified in two metabolic sub-groups according to the pathway used to metabolise hexose sugars: homo- and hetero-fermentative (Kandler, 1983), and bacterial isolates from industrial fermented sugarcane substrates, encompasses both homo- and hetero-fermentative *Lactobacilli* (Costa et al., 2008). It was also found that such contaminating bacteria produce both L(+)- and D(-)-lactic acid optical isomers. Some strains produce only the L(+) form, while others only the D(-) form, but the majority of the strains produce different proportions of both isomers.

As lactic acid titer is an indicator of bacterial contamination level, and the enzymatic kit used for this purpose only detects the L(+) isomer, one can expect that the negative impact of contamination is underestimated, since the D(-) isomer is not computed (Costa et al., 2008).

4.2 The importance of yeast glycogen and trehalose during stress conditions

As said before, yeast viability is of great importance in a Melle-Boinot-based process, since cells will be re-used in subsequent fermentation cycles.

Yeast cells, when fermenting in stressful conditions, usually show drops in cell viability, increased glycerol formation, lower biomass yield, and also diminished levels of storage

carbohydrates, such as glycogen and trehalose. All these parameters are extremely helpful indicators that can be used to select new tolerant strains. Hence, glycogen and trehalose [the two major storage carbohydrates in *S. cerevisiae*, accounting to up 25 % in yeast biomass (w/w dry weight)] have been involved with tolerance towards several stresses (Attfield, 1997; D'Amore et al, 1991; Parrou et al., 1997; Singer & Lindquist, 1998).
During fed-batch fermentation, trehalose and glycogen exhibit great variations. These two compounds are initially degraded in response to increased sugar levels in the medium, and restored when sugar levels decline towards the end of the fermentation (Alves, 2000). The levels of these reserves are higher at the end when compared to the beginning of the fermentation. Therefore, a significant amount of sugars are stored as glycogen and trehaloseat the expense of ethanol production. Fortunately, high levels of carbohydrate reserves are of paramount importance for yeasts to withstand the stressful acid washing treatment, imposed by the industrial process. During treatment, part of the glycogen and trehalose are dissimilated through glycolysis, and ethanol is produced during the acid treatment with no sugar in the medium (Ferreira et al, 1999). At the end of the acid treatment, the levels of these reserves are adequate to guarantee high viability. It is believed that the high tolerance of industrial strains is partially explained by their higher levels of glycogen and trehalose when compared with less persistent strains.

4.3 The starter strain and the competition with indigenous *Saccharomyces* during fermentation

Molecular techniques, such as karyotyping and PCR-fingerprinting, have been used to monitor yeast population dynamics during industrial fermentations.
At the beginning of the 1990's, Basso et al. (1993) verified - for the first time - that some traditionally starter strains (baker's yeast and other two *S. cerevisiae* strains TA and NF), were all replaced by indigenous (contaminating) yeasts in a period of up to 40 days of recycling. This study was performed in 5 distilleries at Sao Paulo State (Southeast region of Brazil) during two crop seasons (1991-1993). In fact, a succession of different indigenous *S. cerevisiae* strains was detected all over the fermentation seasons in the majority of distilleries. Although starter strains were not able to persist during the cell recycling, it was shown that a wild strain (JA-1), formerly isolated from one of the distilleries, was capable to dominate when re-introduced in the process. This was an important indicative for selecting strains from the great biodiversity found in distilleries. Lately, it was verified in a universe of up to 70 distilleries, that baker's yeast were rapidly replaced by wild strains in a very short period (from 20 to 60 days) of cell recycling (Basso et al., 2008). During this study, it was also demonstrated that no strains (from brewing, wining, distilling) other than those isolated from the industrial process had the capability of being implanted in the distilleries investigated.
Da Silva et al. (2005b) employed PCR-fingerprinting as a method to illustrate yeast population dynamics during industrial fermentation. It was again showed that indigenous strains replaced commercial starter strains during the recycling process. Using this technique coupled with physiological assays it was possible to isolate an indigenous strain (JP1) to be used as starter culture for Northeast distilleries (Da Silva et al., 2005a).
A varying proportion of non-*Saccharomyces* strains contaminates the fermentation step. It was found that *Dekkera bruxellensis, Candida tropicalis, Pichia galeiformis, Schizosaccharomyces pombe* and *C. krusei* are major contaminants in acute contamination episodes, being responsible for decreased ethanol yields. Only few distilleries (< 5 %) located at Central and South-eastern regions of Brazil (responsible for the majority of the ethanol production) exhibits

contamination by non-*Saccharomyces* yeasts (Basso et al., 2008). On the other hand, ca. 30 % of distilleries located at the Northeast region of the country suffer with this special contamination (Basillo et al., 2008).

4.4 Selection of suitable strains from the biodiversity found in distilleries

By monitoring yeast population dynamics during industrial fermentations, dominant and persistent indigenous strains have been selected and employed as starter strains.

One example (mentioned above) is the case of strain JP1. It is a highly dominant strain that displays stress tolerance towards acidic pH, high ethanol levels and high temperature. Moreover, it presents fermentative performance similar to other commercial industrial strains. This strain was re-introduced as starter in one of the distilleries it was isolated, and the population dynamics of this plant was followed on a monthly-basis for two consecutive production seasons. It was found that the strain was able to dominate the yeast population while conferring high ethanol yields (> 90%) during this period (Da Silva et al., 2005b).

The most extensive study on yeast population dynamics was performed by Basso et al. (2008), with a monthly yeast sampling scheme from a universe of 70 distilleries, responsible for most of the produced Brazilian ethanol and covering a period of 12 years. During this period, strains with prevalence (which dominate the yeast population in fermentation) and persistence (present along a 200-day season) were identified by karyotyping, isolated and screened in laboratory trails for desirable fermentation traits (no flocculation, low foam formation, high ethanol yield, low glycerol formation, high viability during recycling and high cellular glycogen and trehalose content). As soon a promising strain was identified, it was propagated in laboratory and re-introduced in many distilleries (up to 54, depending on the season) and followed by karyotyping. Along the study, up to 14 selected strains were re-introduced in industrial processes. Some of them (as PE-2) were followed during 10 years in different distilleries, comprising different regions, different processes (fed-batch or continuous), different substrates (molasses/juice), and thus encompassing great variations.

Most of the evaluated strains assessed were not able to permanently perpetuate among yeast population. On the other hand, some strains were able to dominate the fermentation population for a couple of seasons. Probably, variations in process conditions, climate, substrate, etc would be responsible by the lowered capacity of implantation of these strains.

Few strains were able to persist in many distilleries for many seasons. PE-2 and CAT-1 strains showed the highest implantation capabilities. These strains could be implanted in 51 to 58 % of the distilleries where they were introduced. They also presented higher competitiveness in relation to contaminating strains, representing an average of 45 to 54 % of yeast biomass during the fermentation season. In some distilleries, these strains represented the total biomass of the reactor during the whole season (more than 200 days of recycling). Due to these remarkable traits for an industrial fermentation, CAT-1 and PE-2 are the most used starter strains, representing today 80 % of the commercialized active dry yeast for fuel ethanol in Brazil. They are annually used in more than 200 distilleries, responsible for 60 % of the total country's ethanol production.

Even though selecting indigenous strains is considered an attractive strategy to guarantee high product yields and population homogeneity during industrial fermentations, this approach is laborious and not always successful. As appointed by Basso et al. (2008), after a 12 years yeast selection program, among 14 selected strains exhibiting high performance in laboratory trails, only few presented high implantation capability when re-introduced to the industrial process.

Probably, laboratory-screening procedures do not simulate all the stressing agents faced by the yeast in industrial-scale process. Other important factor could be the variations that occur in industrial fermentations from one season to another. It is worthwhile to point that each distillery has peculiar process features, imposing different stresses with different magnitudes to the fermenting yeast. This may explain that a given strain performs well only in a few distilleries (sometimes in only one), and just for one or a couple of seasons.
In conclusion, it can be said that any selected strain must be evaluated in industrial conditions in as many as possible distilleries for many seasons. The most important and rare attribute of superior yeast starters is the implantation capability, since good fermentation profile (at laboratory screening experiments) is more easily found among indigenous and even laboratory strains.

5. Future trends and concluding remarks

It can be foreseen that bioethanol production is expected increase continuously in Brazil, due to the growing investments in this field. New distilleries are being built, resulting in almost 400 operating plants all over the country (MAPA, 2011). They will be producing near 30 billion liters of ethanol in the next season. Although bioethanol production in Brazil is considered a mature process, there is plenty room for improvements.
The current broad interest for the use of very high gravity (VHG) fermentations in the industrial scenario is mainly focused in reducing production costs. It is also expected that this technology will bring benefits to the overall environmental sustainability of the process by decreasing water and energy consumption. Thus, it continues being possible to increase the efficiency of first generation fuel ethanol process by embracing this technology. Fermentations resulting in high ethanol titers would not only benefit the energy balance, but would also result in a significant reduction of vinasse volume. There will be a great economical and environmental impact due to reduction costs of vinasse transportation and application as fertilizer in sugar cane plantations. Besides that, higher ethanol levels would repress bacterial growth during fermentation and for that reason, decrease antibiotics usage for controlling such contaminations.
Despite all these advantages, the implementation of VHG technology in the Brazilian industrial scenario is limited by the availability of very high ethanol tolerant strains. It is expected that for VHG fermentations, substrates will be formulated with more molasses, where sugars are in a concentrated form. As a result, apart from enduring ethanol stress, yeasts will be subjected to others stresses from molasses.
The high international sugar price is leading sugar industry to prioritize sucrose production and more molasses will be generated. Molasses will also be more exhausted (*i.e.* possessing a lower sugar concentration in relation to total solids) and undoubtedly will exert a more pronounced toxic effect upon yeast fermentation. The new yeast strains must also be able to cope with high molasses content substrates, even operating with normal ethanol titers.
Sugar cane bagasse is considered a promising feedstock for second generation ethanol. Nevertheless, the produced ethanol from this lignocellulosic by-product must prove to be more advantageous than the use of bagasse as fuel for steam generation used for milling, heating, distilling, and electricity co-generation.

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This book aspires to be a comprehensive summary of current biofuels issues and thereby contribute to the understanding of this important topic. Readers will find themes including biofuels development efforts, their implications for the food industry, current and future biofuels crops, the successful Brazilian ethanol program, insights of the first, second, third and fourth biofuel generations, advanced biofuel production techniques, related waste treatment, emissions and environmental impacts, water consumption, produced allergens and toxins. Additionally, the biofuel policy discussion is expected to be continuing in the foreseeable future and the reading of the biofuels features dealt with in this book, are recommended for anyone interested in understanding this diverse and developing theme.

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