# Bioethanol – What Has Brazil Learned About Yeasts Inhabiting the Ethanol Production Processes from Sugar Cane?

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## 1. Introduction

According to Datagro (the largest Brazilian sugar and ethanol consulting firm), Brazil will produce nearly 25.3 billion liters of ethanol in the year 2011 (UDOP, 2011). In spite of the astonishing amount, the country does not figure as the world's largest producer, but certainly the bioethanol produced in Brazil stands out in the worldwide clean energy scenario. Ethanol is classified by the US Environmental Agency as an advanced biofuel, since it is capable of reducing greenhouse gas emissions up to 61% when compared to gasoline (UNICA, 2011). This significant production started in the 1970s, leveraged by the first oil crisis. The Brazilian government launched the Proálcool (National Alcohol Program). The required feedstock, sugar cane, has always been plentiful in a country with sugar production tradition. Many plants installed for sugarcane production already had fermentation units to treat molasses, a sugar manufacturing effluent. The government offered incentive to the creation of autonomous distilleries (using sugarcane juice as the only feedstock).

In 1986, out of the 758,965 passenger vehicles manufactured in Brazil, 697,049 were fueled by bioethanol. The end of government incentives to bioethanol production and Brazilian consumers' disbelief in face of bioethanol scarcity in fuel station pumps, associated with the reversion of the worldwide oil crisis, changed the Brazilian scenario. In 1998, out of the 1,389,958 passenger vehicles manufactured in Brazil, only 1,224 were fueled by bioethanol (UNICA, 2011). It seemed as the end of the bioethanol as an alternative fuel source. Many autonomous distilleries were converted into sugar factories, fermenting molasses only as an alternative for effluent treatment. Others were simply closed.

In the 1990s, the eminent global warming threat awakened the world to the need to promote the use of renewable fuels. Ethanol production was quickly resumed in Brazil. In 2001, 16 billion liters of ethanol were produced in the country, and in 2009 this figure escalated to 27 billion liters (UNICA, 2011). In the 1990s, the flex-fuel car (running on ethanol or gasoline)

was launched for consumers of passenger vehicles. In 2009, Brazil produced 2,874,077 passenger vehicles, 92% of which were flex-fuel.

The benefits of the introduction of Proálcool were not limited to give highlight to Brazil as a producer and consumer of clean energy. Those benefits extended to research centers and public and private universities. Today, 35 years after the introduction of Proálcool, Brazil has excelled in different areas of knowledge comprising the sugar and ethanol sector. Average agroindustrial yield grows 3.7% a year in Brazil, and 60% of this figure is a result of research and development originated from science produced in the country (DATAGRO, 2009). This research comprises the study of yeasts inhabiting alcoholic fermentation processes.

Brazil has been producing ethanol since the 1930s, and at that time both this product and sugarcane liquor were produced from spontaneous fermentation. Spontaneous fermentation does not make use of an inoculum, and fermentation takes place by the action of naturally inhabiting yeasts in sugarcane fields that are introduced in the process by sugarcane juice. In 1935, Brazil started to use in its ethanol fermentation processes yeasts referred to as pure, that is, purchased by the manufacturer and with the required characteristics for the industrial alcoholic fermentation process (Amorim *et. al.*, 2005).  The Sugar and Alcohol Institute (IAA) reports to ethanol producers "*Everyone is aware that yield losses faced by ethanol industries have reached appalling figures. These losses are mostly originated from spontaneous fermentation with impure wild yeasts which act on musts highly contaminated by competing microbes against which industry workers, unaware of the modern methods of rational fermentation, do not know what to do in order to weaken their virulence*". The author of this report could not have guessed that in the year 2011 yeasts referred to as "wild" and consequently harmful to the process are currently being qualified as indigenous and are largely propagated to be used in Brazilian fermentors.

Argueso and Pereira (2010) suggest the use of yeasts isolated from Brazilian ethanol fermentation as a biological platform for application of a wide range of new biorefining technologies. According to these authors, these yeast strains present heterogeneous genomic architectures and establish a strong connection between this genomic complexity and its adaptation to the industrial environment.

Regardless of the time or the fermentation method, the yeast responsible for the transformation of cane sugar or molasses belongs to the *Saccharomyces* genus. *Saccharomyces* is the widely used yeast genus in the industry of fermented products which uses alcohol as final product, whether for fuel or production of alcoholic beverages. This microorganism is mostly indicated for this purpose because it gathers all attributes required for conducting the alcohol production process. The ability to rapidly turn sugars into ethanol, high tolerance to the formed product, osmotolerance (tolerance to great temperature variations), and cell activity in acid environments are the main desirable traits for an industrial strain. All of these attributes are found in representatives of the *Saccharomyces* genus (Andrietta *et al*, 2007).

In Brazil, most industrial processes installed for biofuel production use yeast cell recycling, thus allowing an operation with high cell concentration in the process. This strategy makes fermentation fast and promotes competition between contaminating bacteria and yeast, which is favorable to yeast cells. High conversion speed of sugars into ethanol, though desirable, causes some problems, such as a great amount of heat generated per time unit. This heat should be removed, otherwise, temperature in fermentors may reach levels that are physiologically harmful to yeast cells. Heat is

removed with the installation of plate heat exchangers. They are installed outside fermentors. Cooling water originates from a closed circuit and heat is cooled by cooling towers. Since Brazil is a tropical country, the temperature of wet bulbs in sugarcane producing areas range throughout the year from 24 to 27°C, which allows cooling water temperatures from 27 to 30°C, which in turn keep temperatures in fermentors from 31 to 34°C. This process characteristic becomes the first strong factor in yeast selection, leading to the installation of strains capable of developing at higher temperatures. Although these strains develop well at these temperatures, they become more sensitive to ethanol concentration. From 11°GL in temperatures practiced in Brazilian industries, yeasts already compromise their performance.

Even though cell recycling is a requirement in fermentation industry, it is important to highlight that along with yeast cell recycling, this type of operation also recycles contaminating bacteria.

These bacteria are usually acid producing Gram-positive rods, which develop well in pH near 5.0. Aiming to decrease the action of these bacteria, Brazilian distilleries treat recycled yeast with sulphuric acid in pH between 2.0 and 2.5 at times from 1 to 2 hours. Low pH in inoculum makes possible to keep fermentation pH between 3.8 and 4.2, creating an environment less favorable to the growth of bacteria found in the medium. This acidified medium also becomes a selection factor of yeast population in the process. Thus, only yeast strains which develop better in mediums with pH values within this range are capable of dominating the process.

## 2. Bioethanol fermentation processes

Alcoholic fermentation processes in Brazil are conceived similarly to those found in other countries. They are made up of three basic units: fermentation per se, cell separation unit and recycled yeast treatment unit. Fermentation per se is constituted of fermentors, where sugars are converted into ethanol. These fermentors are usually built of carbon steel, with lining properly painted to prevent corrosion and make walls less wrinkled, thus making cleaning easier. They are equipped with external heat exchangers and a shaking system that uses the kinetic energy from the fermentation medium, which is recycled by the heat exchangers. They are closed fermentors with a cleaning system. The conversion that takes place in these fermentors is fully anaerobic and gases produced in fermentation are sent to a washing column for recovery of ethanol found in them. The cell separation unit is made up of centrifuge separators. The fermentation medium containing between 10 and 13%(v/v) of yeast cells is sent to the centrifuges, which generate two product lines, the centrifuged wine, whose cell concentration ranges between 0.2 and 1%, and the yeast cream, whose cell concentration is between 60 and 75%. The centrifuged wine is sent to a lung tank of the distillation devices and the cream is sent to treatment tanks. The treatment unit consists of well-shaken tanks, equipped with an aeration system and built the same way as the fermentation tanks, that is, in carbon steel and with lining properly painted to avoid corrosion and make walls less wrinkled, so as to make cleaning easier. Treated water is added to the yeast cream in this tank with a dilution for cell concentration between 30 and 40% (v/v), then adding concentrated sulphuric acid for pH adjustment in the range from 2 to 2.5. Yeast cells remain in this treatment for 1 to 2 hours. The treated yeast cells return to the fermentors after treatment.

Although the fermentation processes have the same processing stages, they may differ in the operation method, with the fed-batch and continuous processes.

## 2.1 Fed-batch fermentation process

The fed-batch process presents basically the following characteristics: the treated ferment (inoculum) is transferred from the treatment tank to the fermentors through pumping. With transfer concluded, feeding of substratum to be fermented starts until the final fermentor volume is reached. After fermentor is filled, sugar draining is expected. After sugar is converted into alcohol and other products, the fermented medium is sent to the centrifuges where yeast cells are separated from the wine and sent to treatment tanks, where they are diluted and acidified in order to start a new cycle.

The fed-batch fermentation process was conceived to be used in cases where the agent microorganism is submitted to sharp inhibition by the substratum. In this case, in order to obtain higher final product concentrations, substratum is added to the fermentor in a controlled way so as to decrease this inhibition. In general, in plants using this type of process, substratum feed curves are exponentials and aim at keeping substratum in fermentation medium constant and lower than inhibiting concentration. In Brazilian bioethanol producing distilleries, feed curves differ from this standard, and are usually constant throughout the filling time. This happens because the yeast strains used in these processes present little inhibition by the substratum. These strains develop in concentrations from 65 g/L of glucose and 70 g/L of fructose, figures hardly achieved in ordinary operations. Low concentrations of these substrata significantly affect fermentation speed, characterizing the limitation effect by the substratum presented by these microorganisms. For that reason, higher fermentation speeds are obtained when the fermentor is fed quickly, due to the higher glucose and fructose concentration in the medium, without which ethanol yield would be affected (Ferreira, 2005). On the other hand, high fermentation speed leads to higher carbon dioxide production, which increases the foam formation and, consequently, the consumption of antifoam and the generation of heat, which to be removed, require heat exchangers with larger thermal exchange area. These facts make fermentor fill-up time in Brazilian industrial units take approximately 75% of the total fermentation time, which allows better distribution of the heat produced during the fermentation time.

## 2.2 Continuous fermentation process

As for the fed-batch evolution process, the continuous fermentation process is usually conceived with 4 or 5 fermentors connected in series, wherein a cultivation medium and treated yeast cells are added simultaneously and in a continuous and controlled manner in the first fermentor of the system. The purpose of the use of this configuration is to approach the system's behavior to a piston-flow fermentor, thus minimizing the inhibition effect by the product (ethanol) in the transformation speed of sugars into ethanol. The amount of TRS (Total Reducing Sugars) fed into the first fermentor is proportional to the amount of yeast cells, keeping an average rate of 3.5 g of TRS / g cell mass (dry base). This control is important to keep the conversion rate of each fermentor, providing a total fermentation time between 7 to 8 hours depending on the strain used and the feedstock employed. The fermentation medium flows from one fermentor to the other until it reaches the last one, from where it is taken to centrifuge separators, where ferment is separated from wine and sent to treatment tanks. The treatment unit of this type of process is made up of 3 tanks connected in series. Sulphuric acid, water and yeast cream are mixed in a small tank, from where the acidified ferment follows to the treatment tank. This material flows from one tank

to the other until it reaches the third tank, from where it is taken to the first tank of the system. Ferment treatment is carried out in three tanks with the purpose of distributing its volume, making the size of the tanks more suitable to the plant layout.

## 2.3 Comparison between the two types of process

Since it is a process that works in stationary state, equipment use rate in continuous fermentation is 100%. This guarantees higher productivity in the process. The occupation rate of the batch system is compromised as a result of operations such as: inoculum load, centrifugation of the fermented medium and cleaning of fermentors.

With the increase in productivity, it is possible to produce the same amount of ethanol of a fed-batch plant, with significant reduction in equipment and physical area. As a result of this reduction, continuous fermentation plants have reduced installation cost when compared with the fed-batch system. The initial investment for installation of a continuous fermentation process ranges from 60 to 70% when compared with the total installation cost of a fed-batch fermentation unit with the same capacity.

As for automation of the continuous fermentation process, since it works in stationary state, it is simpler and more economical when compared with the fed-batch process.

On the other hand, fermentor cleaning is easier in fed-batch processes. In this process, the fermentor is completely emptied at the end of the cycle, which guarantees more efficient equipment cleaning. All yeast cell mass from fermentor centrifugation is sent to juice treatment. Since 100% of inoculum undergoes acid treatment, ferment sent to the next fermentation cycle is cleaner when compared with ferment from continuous fermentation. For this type of operation, only 15% of yeast cells in process are being treated, returning to the fermentor, which is never emptied. This fact contributes to an increase in bacteria contamination in continuous processes. However, with the new cleaning systems of line, fermentors, treatment tanks, heat exchangers, it is possible to keep this contamination under control and no significant difference in ethanol yield values has been observed between the two processes when properly operated.

## 3. The *Saccharomyces sensu strict* strains isolated from Brazilian industrial units

According to Basso *et al.*, (2008) in 2006 there were 329 bioethanol plants in Brazil, and 190 distilleries opted for starting their processes with selected yeasts. In this specific case, selected yeasts are understood as those isolated from industrial fermentation processes. These yeasts are indigenous of their environments, and they all belong to the *Saccharomyces sensu stricto* group. Four strains (CAT1 PE2, SA1 and BG1) are being produced in large scale and commercialized to be used as inoculum to start up alcoholic fermentation processes. The names of these strains correspond to their original units. Thus, strain CAT 1 was isolated at the Catanduva unit, belonging to the Virgolino de Oliveira S/A Açúcar e Álcool Group, whereas PE2 is originally from da Pedra Agroindustrial S/A unit, SA1 from Usina Santa Adélia S.A unit and BG1 from Usina Barra Grande de Lençóis S/A. All these units are located in the State of São Paulo. Some units chose to use a mix of these four strains to start up their processes. Figure 1 presents the karyotype profile of these four strains.

This information is not capable of revealing the fermentative performance of the strains, but it is an efficient tool to evaluate the permanence of these strains in industrial processes.

From this profile, it is possible to separate the different strains present in a sample of a fermentation process. Information concerning indigenous yeasts isolated from Brazilian alcoholic fermentation processes is still scarce, but Argueso et al. (2010) describes the complete genome of a haploid descendent of the PE2 strain and reports the following: "*Here we report the molecular genetic analysis of PE-2 derived diploid (JA Y270) and the complete genome sequence of a haploid derivative (JA Y291). The JA Y270 is highly heterozygous (2 SNPs/kb) and has several structural polymorphisms between homologous chromosomes. These chromosomes have breakpoints within repetitive DNA sequences. Despite its complex karyotype, this diploid, when sporulated, had a high frequency of viable spores. Hybrid diploids formed by outcrossing with the laboratory strain S288c also displayed good spore viability. Thus, the rearrangements that exist near the end of chromosomes do not impair meiosis, as the not span regions that contain essential genes. This observation is consistent with a model in which the peripheral regions of chromosomes represent plastic domains of the genome that are free to recombine ectopically and experiment with alternatives structures. We also explore feature of the JA Y270 and JA Y291 genomes that help explain their high adaptation to industrial environmental, exhibiting desirable phenotypes such as high ethanol and cell mass production and high temperature and oxidative stress tolerance*".
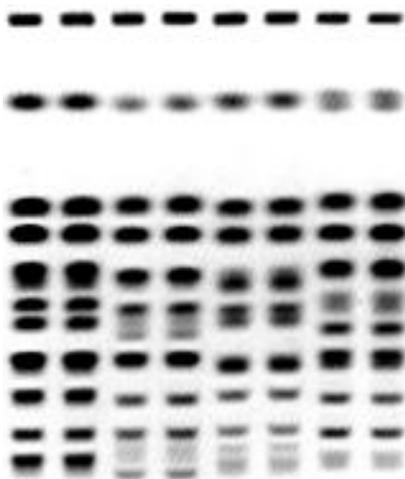


Fig. 1. Karyotyping profile of strains (from left to right): row 1 and 2, strain SA1; row 3 and 4, strain PE2; row 5 and 6, strain CAT1 and row 7 and 8 strain BG1

Stambuck *et al.*, (2011) evaluated, by using microarray-based comparative genome hybridization (aCGH), five different yeast strains (BG-1; CAT-1 PE-2, AS-1 and VR-1) isolated from Brazilian ethanol fermentation processes. Their results suggest that with regard to the reference laboratory *S. cerevisiae* strain, S288C, all five fuel strains showed significant amplification of the telomeric SNO and SNZ genes, which are involved in the biosynthesis of vitamins B6 (pyridoxine) and B1 (thiamin). These authors report that the increase in the number of copies of these genes confer on lab yeast strains the ability to grow more efficiently under the repressing effect of thiamin, especially in the absence of pyridoxine and in high sugar concentration, suggesting that the these gene amplifications provide an important adaptive advantage under the industrial fermentation conditions in which the yeast are propagated.

Although few works present the genetic and technological characteristics of yeasts from Brazilian processes, there is already a consensus that these yeasts are robust and, consequently, are deemed as suitable to be domesticated in order to make them easily handled. Galzerani (2010) domesticated an isolate of the PE-2 industrial strain. This author states that at the end of the domestication process, a strain presenting auxotrophy to uracil was obtained, since the gene URA3 was deleted from the genome of the diploid strain. According to this author, the auxotrophic yeast generation will facilitate the application of genetic engineering techniques.

As for the technological properties of the yeasts, some works have been carried out with yeasts isolated from fermentation processes. Tosetto (2008) studied the influence of organic substances known to be present in sugarcane molasses on the performance of two industrially used yeast strains. One of the strains tested was the Y904 (Mauri – Brazil) which, in spite of being used as inoculum by some industrial units, is a strain developed to be used in bread making. Another strain used was SA1. The study was carried out using eight fermentation cycles in sucrose-base synthetic medium with addition of the following acids: caffeic, lactic, syringic, vanillic, butyric, acetic, formic and HMF(hydroxymethylfurfural). The results suggest that there are behavioral differences between the two strains. The performance of the SA1 strain was compromised by the presence of formic and gallic acids whereas the performance of the Y904 strain was affected by the presence of acetic and butyric acids, as well as by HMF. The presence of lactic acid was a negative interference in the yield and productivity parameters for the two strains studied. Observing these data, it is possible to infer that the presence of a certain substance in the feedstock content of a unit may be a strong microbiota selection factor in fermentation tanks.

Alcarde (2001) studied the flocculation of yeasts cultivated in the presence of bacteria known to promote this phenomenon (*Lactobacillus* and *Bacillus*). The data presented show that the PE2 yeast strain is less sensitive to the flocculation phenomenon than the VR1 strain (yeast strain isolated from the process at Santelisa Vale Bioenergia S/A – Morro Agudo – São Paulo, Brazil) when in the presence of ethanol (7 to 9%).

Steckelberg (2001) characterized strains isolated from nineteen Brazilian alcoholic fermentation processes. This work was carried out under standardized conditions so as to guarantee the same cultivation conditions for all strains, which allows the comparison of yeasts between them. These data are presented in the following table, demonstrating the diversity between the strains isolated from different units. Table 1 presents the kinetic parameters, trehalose and protein percentages, respectively.

The data presented by the author elucidates the diversity of yeast strains inhabiting the Brazilian industrial processes. Even considering all these variations in the data obtained for these strains, it is important to highlight that none of these units presented yield and productivity problems with regard to the presence of these strains as dominant in their processes.

The values concerning all parameters compiled are significantly different and lead to the inference that, although they are yeasts belonging to the *Saccharomyces sensu strict* group, they differ in terms of strain. In theory, strains producing larger amounts of $Y_{x/s}$ cell shave higher chances of dominating fermentation processes. The analysis of the data in Table 1 shows that, under industrial conditions, not all isolated strains present high values for that parameter. The $Y_{x/s}$ of dominating yeasts in their original processes vary up to 25%. A

similar variation is observed for the *μmax* value, a parameter which is also a strong influence on the dominion of a strain in the process, since the higher the parameter, the faster the strain develops. Thus, only strains presenting high values for this parameter were expected to be able to dominate the processes, but this fact has not taken place, and this value ranged from 0.35 to 0.60 h-1. Considering trehalose, which is intimately related to external conditions, such as nutrient deprivation, temperature variation, high ethanol concentrations and osmotic pressure (Lilie, 1980), it is possible to assume that the processes where the strains were isolated presenting high concentrations of these sugars in their cell composition, were operating under extreme conditions, which may be temperature, osmotic pressure or high alcoholic contents.  These yeasts were only capable of surviving these conditions because they were able to accumulate this sugar as a protection factor. Some strains present values near 6%, whereas others are lower than 0.02% in their cell composition. Following this theory, strains presenting lower trehalose levels in their cell compositions are probably originated from processes not operating under extreme conditions.

| Strain origin (industrial unit) | Yx/s | VCS | NCO | μmax | ∅ | Yp/s | % Trehalose | % Protein |
|---|---|---|---|---|---|---|---|---|
| Alvorada | 0.0436 | 5.8128 | 98.31 | 0.5136 | 2.6487 | 0.4712 | 4.07 | 42.02 |
| Unialco | 0.0401 | 5.4880 | 96.58 | 0.4867 | 2.3798 | 0.4512 | < 0.02 | 42.63 |
| Diamante | 0.0490 | 5.8885 | 99.59 | 0.3756 | 2.6528 | 0.4617 | 4.03 | 42.18 |
| Diana | 0.0409 | 6.5118 | 99.64 | 0.4172 | 2.8080 | 0.4502 | 6.33 | 42.84 |
| Jalles Machado | 0.0474 | 5.8823 | 99.48 | 0.4129 | 2.6102 | 0.4589 | 2.07 | 44.00 |
| Junqueira | 0.0401 | 5.8808 | 99.46 | 0.3809 | 2.6558 | 0.4719 | 1.27 | 44.74 |
| Goiasa | 0.0463 | 5.5473 | 99.64 | 0.4578 | 2.4686 | 0.4607 | 6.17 | 44.93 |
| Vale Rosário | 0.0391 | 5.5216 | 99.17 | 0.3537 | 2.3267 | 0.4399 | 0.45 | 48.79 |
| Bonfim | 0.0452 | 5.6389 | 99.24 | 0.5439 | 2.5122 | 0.4610 | 1.74 | 40.53 |
| Costa Pinto | 0.0469 | 5.5467 | 99.63 | 0.4994 | 2.3839 | 0.4466 | 1.52 | 45.40 |
| Guarani | 0.0342 | 5.1921 | 93.26 | 0.5695 | 2.2160 | 0.4472 | 1.94 | 48.24 |
| Andrade | 0.0404 | 5.5444 | 99.59 | 0.5694 | 2.4629 | 0.4608 | 5.61 | 44.41 |
| Dacal | 0.0368 | 5.5467 | 99.63 | 0.6045 | 2.4898 | 0.4660 | 1.76 | 45.07 |
| Dacalda | 0.0489 | 5.5467 | 99.63 | 0.5985 | 2.5167 | 0.4721 | < 0.02 | 42.14 |
| Alcoeste | 0.0424 | 5.5615 | 97.88 | 0.5857 | 2.3967 | 0.4458 | 0.71 | 44.41 |
| Corol | 0.0466 | 6.5118 | 99.64 | 0.5640 | 2.8419 | 0.4563 | 5.13 | 39.33 |
| Barra Grande | 0.0399 | 5.6500 | 99.58 | 0.5767 | 2.4332 | 0.4421 | 2.78 | 47.25 |
| Santa Cruz | 0.0450 | 6.5118 | 99.64 | 0.4316 | 2.8420 | 0.4563 | 1.94 | 44.84 |
| Clealco | 0.0440 | 5.8000 | 99.50 | 0.5000 | 2.5000 | 0.4600 | 4.23 | 45.20 |

Table 1. Kinetic parameters, yield and productivity of 19 isolated strains, wherein: Yx/s = g produced cell mass (dry mass)/g substratum; VCS = g substratum consumed/Lxh; NCO = % substratum conversion level; μmax = maximum specific growth speed (h-1); ∅ = g ethanol produced/Lxh; Yp/s = g ethanol produced/ g substratum, % de trehalose (g/100g of dry mass) and protein (g/100 grams of dry mass)

As for protein concentration, it is possible to assume that yeasts presenting higher concentrations of this compound in their cell compositions were isolated from processes using a must with greater nutrient availability. In general, fermentations carried out with substrata at lower protein concentrations do not influence on yeast performance with regard to ethanol production, but the fact that most Brazilian units sell their surplus yeast, as a protein source for preparation of animal feed, must be taken into account. Thus, units presenting strains with high protein levels obtain a product with higher aggregate value.

The diversity of yeast strains found in processes theoretically equal between themselves - since they originate from the same feedstock, sugar cane, sugar and its byproducts and obtain the same product, ethanol, - suggests that there is not one sole factor responsible for the installation and permanence of a yeast strain in a certain process, but a series of variables which add up to determine the selection and dynamics of the yeast population in the process. This dynamics is unique for each one of the processes.

These data elucidate the diversity of strains inhabiting a restrictive process, such as fermentors, and suggest that this artificial ecosystem is an inexhaustible source of microorganisms, which present particular characteristics and may be used to obtain products with higher aggregate power.

## 4. Population dynamics of yeasts in fermentors

Even though the selected yeast strains are more suitable when compared with the bread-making strains, few industrial units end up the season with the same strain they started. Based on the practical data, PE2 seems to be the most persistent strain among the indigenous strains used. The reasons for this strain to be different from the others are still unknown, but high cell yields must be taken into account for this strain. Amorim *et al*., (2008),studied the permanence of indigenous strains in industrial processes during 12 harvest seasons. The results presented show that the PE2 strain was capable of remaining in 58% of the distilleries where it was used as inoculum at the beginning of the season.

Another important point, which must be highlighted in the population dynamics in fermentors  refers to the number of strains in process. One sole yeast strain is rarely observed in process samples. The presence of two or more strains is quite common. In most cases, the yeast population in fermentors is made up by a consortium of yeasts. These yeast strains belong to the *S. sensu stricto* group, since no other yeast group is capable of surviving under the extreme conditions found in fermentor environments. Some authors report the presence of non-*Saccharomyces* strains in fermentors. This fact is not surprising since the fermentation must, which is a non-restrictive environment to microorganism growth, carries an indigenous yeast load at levels of about $10^5$ cells/ml.  This fact explains the presence of strains that do not belong to the *Saccharomyces* group in process samples. However, these strains are naturally eliminated.  On the other hand, the indigenous *Saccharomyces* present in the feedstock are those strains that will end up dominating the process, replacing the selected strains used at the start-up. This replacement does not harm the process, since the performance of indigenous strains capable of dominating the process is usually similar to that of strains now referred to as selected, but which used to be native.

A study carried out by Cabrini *et al*., (1999) at a Brazilian distillery producing alcohol for fuel has reported the presence of a diversified yeast flora in sugarcane juice and mash samples. Isolates belonging to the *Saccharomyces, Candida, Torulopsis* e *Rhodotorula* genus have been found in these samples. When the same survey was carried out in yeast samples

undergoing acid treatment to eliminate part of the microorganisms, the *Saccharomyces* genus represented 88% of the isolates. The remaining 12% belong to the *Candida* genus. The presence of these microorganisms is not expected at this stage of the process; however, since this yeast inhabits the raw material, its presence in low concentrations may be explainable in the sample.

## 5. Why feedstock is a determinant factor in yeast population selection in fermentors

The main characteristic of yeast cells inhabiting bioethanol production fermentors is their ability to withstand great biotic and abiotic oscillations, since they are submitted to a process operating uninterruptedly (some Brazilian units operate up to 250 days per season) and in extreme conditions.   In general, this process yeast develops well at high temperatures, low pH, presenting low inhibition by substratum and product and satisfactory ethanol yield values (Yp/s) and low nutritional requirement. In addition to these limitations, it must be capable of withstanding feedstock variations, which are quite a few in an agroindustrial unit such as the sugar and ethanol industry.

Special attention must be given to the type of feedstock used in ethanol production, since it is, in most units, a consequence of the requirements of the producing unit, which is controlled by the world's sugar market. Thus, Brazilian ethanol is produced from a substratum, which may not be standardized so as to meet the needs of the yeast.

The material to be fermented may vary from sugarcane juice itself to molasses and, most of the time, the combination of these two substrata. In terms of sugarcane juice, it may vary depending on the sugarcane variety, the soil where it was planted, the harvest period, crops pests, harvest method (manual or mechanical), microbial load contamination, juice extraction method and others. As for molasses, all factors mentioned as affecting sugarcane juice are added to the operation conditions of the sugar factory, since molasses is a byproduct of the latter. Molasses has different exhaust levels (rate between total reducing sugars concentration and total soluble solids concentration), which is determined by the sugar factory operation method. Molasses differs from sugarcane juice for presenting higher nutrient content. On the other hand, since it is a product resulting from a manufacturing process, where it is exposed to high temperatures, molasses presents a series of secondary products which may interfere in the fermentative process, such as: low-molecular-weight organic acids (lactic, acetic, formic and others), hydroxymethylfurfural (HMF), melanoidines and others. As a rule, fermentations using only molasses in must content present more problems than those using sugarcane juice, mainly for presenting more inhibiting substances and higher osmotic pressure, suggesting that the harmful effects of inhibitors are predominant over the beneficial effects of higher nutrient availability.

## 6. Why indigenous yeasts prevail in fermentors

In a system where the input of contaminating microorganism (yeasts and bacteria) is constant as in alcoholic fermentation processes in Brazilian distilleries, strains originated from feedstock and which dominate the process are usually the ones with the best set of required characteristics for survival in this environment at that moment. Since operation conditions vary from one industrial unit to the other, from one season to the other in the same industrial unit and even in different periods in the harvest season of the same

industrial unit, differences in yeast cell populations between units, from season to season and also along the season are also expected. Considering the operation conditions of bioethanol producing plants, it is easy to understand why it is impossible to choose one sole yeast strain as the most suitable for ethanol production. As far as it is presently known, there is one yeast strain, isolated from a Brazilian alcoholic fermentation process and which has the ability to remain, not always as the dominant strain, in most part of distilleries where it is used as inoculum. This strain is known as PE2. Its ability to dominate the process seems to be connected to the high yield in cell (Yx/s) and high substratum consumption speed this strain presents. Argueso & Pereira (2010) accredit this permanence to the genomic complexity of this strain, which allows it to adapt to the industrial environment.

In order to illustrate the strong influence of the yield parameter in cell (Yx/s) in the process dominance, Figure 1 presents a simulation of the population dynamics of a fermentation process whose initial inoculum was a certain hypothetical strain with kinetic behavior similar to those observed in strains regularly isolated from industrial fermentors. The study has adopted the usual operation conditions of Brazilian industrial units for a fed-batch fermentation process: a fermentor with 100,000 l of useful volume, with yeast inoculum mass from a previous cycle of 2,700 kg (dry base) and 12-hour fermentation cycles. The study assumed the input of indigenous yeast with the same kinetic profile presented by the strain used as inoculum, differing only in the amount of cell yield, 20% higher to the one presented by the strain used as inoculum. It also considered that the feed must introduced in this fermentor carried a load of indigenous yeasts approximately 100 times lower than the yeast used as inoculum (27 kg dry base). In this profile, it was assumed that there was no introduction of new strains as inoculum and no input of a second indigenous strain.
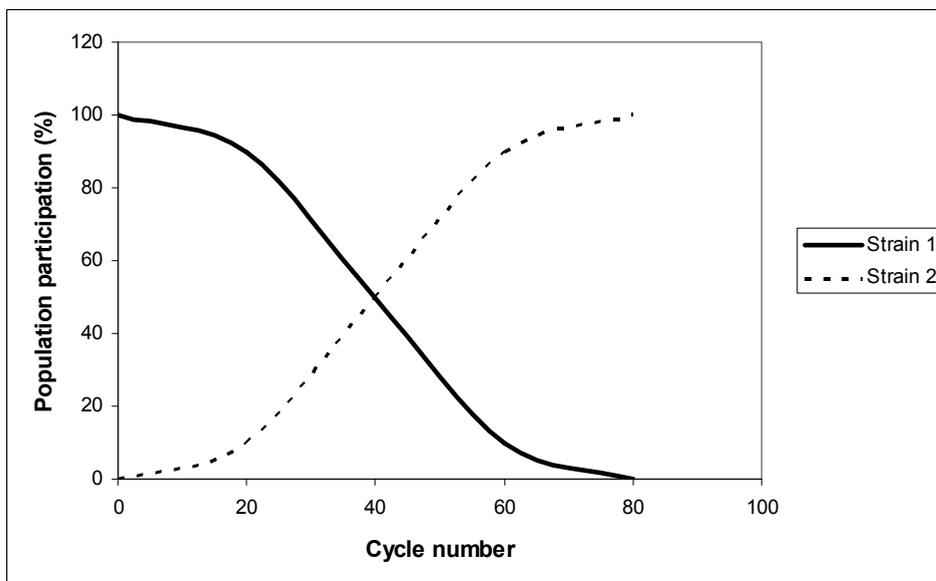


Fig. 2. Population participation profile of two strains with regard to the number of cycles.

The dominance of indigenous strain (yeast 2) in a fermentation process is illustrated in Figure 2. The time for yeast concentration to match the inoculated strain concentration is of 40 cycles, that is, 20 days. On the 70th day of harvest season, which corresponds to 35 days, the indigenous strain fully replaces the strain used as inoculum. These data illustrate the relevance of the cell yield parameter in the definition of the installation, dominance and permanence of a strain in the fermentation process. Thus, yeast strains selected to the used in fermentation units must combine satisfactory characteristics as for the fermentative performance associated to the capacity of presenting high cell yield (Yx/s). The installation and dominance of a certain yeast strain depends on a number of factors, which shall not be assessed apart, but strains presenting high cell yield will certainly be privileged in this dynamics.

The assertions made when using a mathematical model are reinforced when we assess the results obtained in the industrial units. A follow-up of the dynamics of this yeast population carried out in two different industrial units during the 2010 season, which started its processes with the PE2 strain. This strain presents, in lab conditions, $Y_{x/s}$ = 0.0479 grams of dry mass/g of reducing sugar consumed, which is considered high. For comparison purposes, Table 2 presents the values for the four selected commercial strains used in Brazilian distilleries.

| Strain | $(Y_{x/s})$<br>Grams of cells/total reducing sugar consumed |
|--------|------------------------------------------------------------|
| PE2 | 0.0479 |
| CAT1 | 0.0409 |
| SA1 | 0. 0440 |
| BG1 | 0.0463 |

Table 2. Cell mass yield $(Y_{x/s})$ for four yeast strains isolated from Brazilian alcoholic fermentation processes.

The names of the two assessed units were kept confidential, but both units use the fed-batch processes and must with cane molasses base diluted in water as fermentation substratum. Collections were made during the months of April through October, with intervals of 30 ±2 days. Yeast colonies were selected based on their cell morphology in Wallerstein Laboratory Nutrient Agar cultivation medium (WL nutrient medium - DIFCO 0424-17-9) when grown for 7 days at 32ºC. Yeast strains present at concentrations higher than $10^6$ CUF/ml of sample were selected.  Strain differentiation was performed with the use of karyotyping (Andrietta et al., 2008).

Yeast population dynamics during the season, for a certain industrial plant, herein designated Unit A, is shown in Figure 3.

In the first collection, the PE2 strain is the only strain found in fermentors, since it was used as inoculum. After 30 days, this strain is still present in the process, but the installation of an indigenous strain (*Indigenous 1*) was observed. This strain started to dominate the process, representing 62.5% of the total yeast population. In the third collection (approximately 60 days into the season), the presence of the PE2 strain was not observed. At that point, the yeast population is made up of three indigenous strains, *Indigenous 1*, which still dominated the process (59.3%) and two more strains (*Indigenous 2 and Indigenous 3*), which appeared for the first time in the process. In the fourth collection (approximately 90 days into the season),
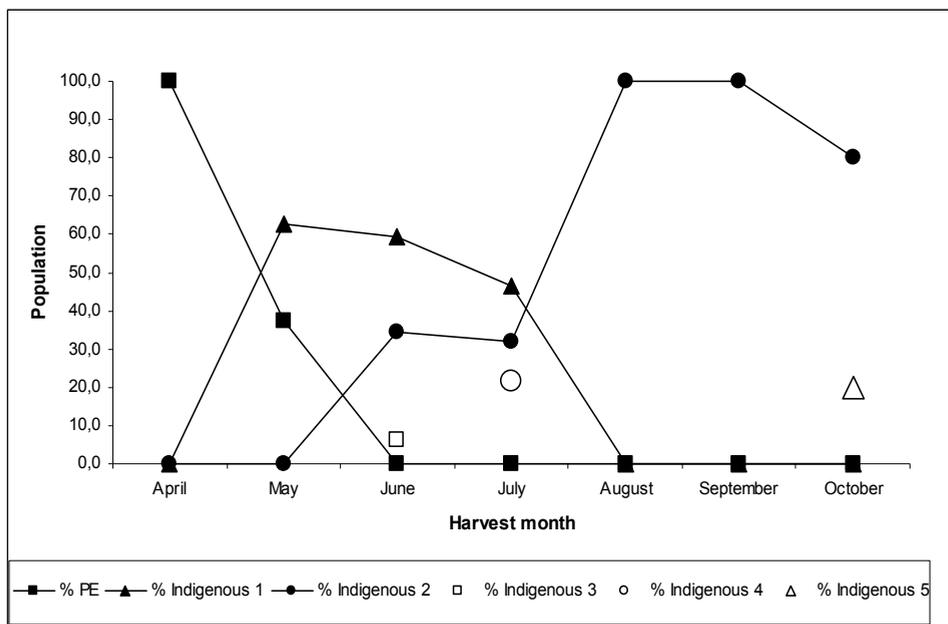
Fig. 3. Yeast population dynamics in industrial unit A

the process kept on operating with three yeast strains in the process, *Indigenous 1* continued to dominate (46.4%), *Indigenous 2* was still present in the sample, but another strain (*Indigenous 4*) came up. *Indigenous 3* strain was no longer detected in the process. In the fifth collection (approximately 120 days into the season), *Indigenous 2* became the only yeast strain in process and thus remained until the sixth collection (approximately 150 days into the season). The seventh and last collection (approximately 180 days into the season) presented yeast *Indigenous 2* as dominant, now inhabiting with another indigenous strain (*Indigenous 5*), which had not been detected in any of the previous collections. Yeast population dynamics during the season of unit A had the presence of six different yeast strains, five of them Indigenous and PE2. *Indigenous 2* was capable of installing, dominating and remaining in the process for the longest time and it was detected in five of the seven collections, predominantly in the three last ones. This strain was present in 120 days out of a total of 180 days. These statements are based on the results obtained from the data; however, some questions remain to be answered: Why hasn't *Indigenous 2* yeast come up right at the beginning of the season? ; Why did this yeast take 60 days after its installation to become dominant? How did this yeast manage to eliminate other process yeast strains and represent 100% of the populations for a 60-day period? Why does *Indigenous 5* yeast install into the process only in the last period of the season? Would it be eliminated should the season extend beyond October, or would it dominate the process in the stead of *Indigenous 2* ? Why do *Indigenous 3* and *4* come up in one collection and are quickly eliminated from the process?

The yeast population dynamics of another assessed unit, herein referred to as B, is presented in Figure 4.
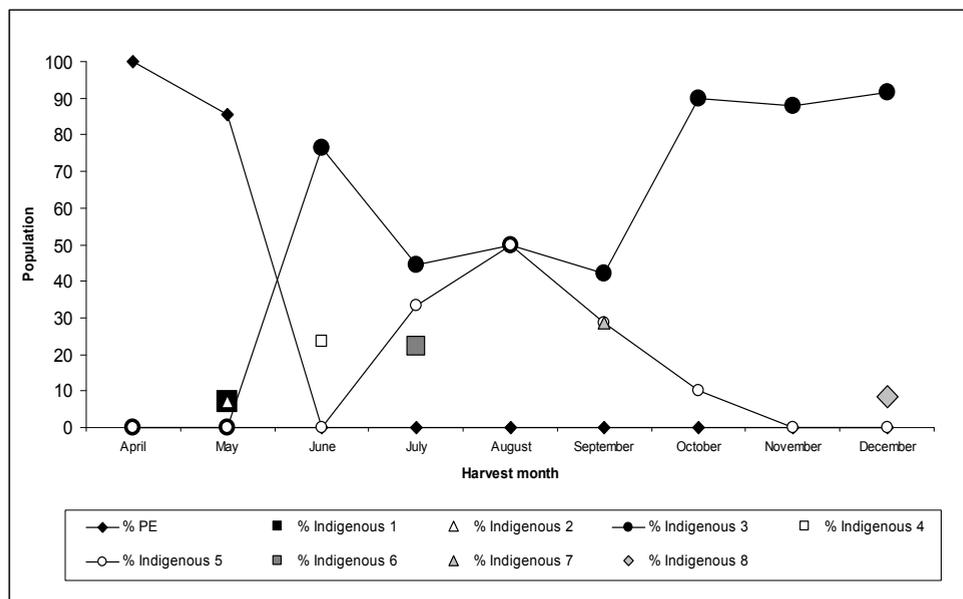
Fig. 4. Yeast population dynamics in industrial unit B

In the first collection, as expected, the yeast population in tanks was 100% of the PE2 strain. After 30 days into the season, PE2 dominated the process, representing 85.6% of the population, but, with participation of two more indigenous yeast strains (*Indigenous 1* and *Indigenous 2*). At 60 days into the season, a completely different scenario was found. PE2 strain was completely eliminated from the process and the two indigenous strains, present in the prior sample (30 days into the season) were also eliminated from the process. The population was now made up of two indigenous strains (*Indigenous 3* and *Indigenous 4*), which completely replaced the PE2 strain within 30 days. *Indigenous 3* presented an aggressive behavior, since it represented 76.5% of the yeast population in the tanks at the first time it was detected in the process. The yeast population present in the tanks in 90 days was made up of three different yeast strains. *Indigenous 3*, which presented as aggressive at the moment of its installation, still dominated, but it did no longer represent 76.5% of the population, but less than half (44%) of the total population. This strain shared the fermentation environment with two other strains (*Indigenous 5* and *Indigenous 6*), which were present for the first time in that process. *Indigenous 4* disappeared from the process from this period on. At 120 days into the season, *Indigenous 3* represented 50% of the population in the fermentors, together with *Indigenous 5*, which also contributed to 50% of the population. At 150 days into the season, *Indigenous 3*, even at a lower concentration, resumed dominance of the process and shared the population with two more strains, *Indigenous 5*, which remained in the process and one new strain observed, *Indigenous 7*. At 180 days into the season, *Indigenous 3* strain once again presented an aggressive behavior and was able to reach 90% of the total yeast strains in the process. *Indigenous 5* strain, even at a low 10% concentration, was able to remain in the process. This same scenario is observed at 210 days into the season, and *Indigenous 3* significantly dominated the process (88%),

although *Indigenous 5* was still present. At the last collection (240 days into the season), the scenario for *Indigenous 3* strain persisted and it represented 91.6% of the population. However, at that time *Indigenous 5* disappeared from the process and a new indigenous strain (*Indigenous 8*) came up. The yeast population dynamics during the season for this unit included the presence of nine different yeast strains, eight of which of *Indigenous* origin and the PE2, which was used as inoculum. The yeast presenting the ability to install, dominate and remain for the longest time in the process was *Indigenous 3*, which was detected in seven out of nine collections. It seems that the presence of this strain was responsible for the elimination of PE2, since the latter came up for the first time in June, with a significant participation of 76.6% of the total population. Although it presented an aggressive behavior, in no period of the season did it represent 100% of the population and, in spite of entering the process at high concentration, it did not maintain this standard throughout the fermentation operation period, and in 90 days (July, August and September) it did not represent more than 50% of the total population. As discussed for unit A, some questions remain to be answered to elucidate the yeast population dynamics in fermentors: Why wasn't *Indigenous 3* strain already present at 60 days into the season? Was this strain really responsible for the elimination of the PE2 strain? Why has this strain lowered its percentage of participants during a period? Why was it in no time able to constitute 100% of the population in the tanks?

Many questions are left unanswered regarding the yeast population dynamics in fermentors used in ethanol production, but based on the results obtained thus far, it is possible to make some statements concerning this dynamics. It will be very difficult to isolate one strain which is able to persist in all, or at least in most, industrial processes. The units rarely work with one sole yeast strain in process. This population is usually made up of two or more yeast strains. This fluctuation is closely connected to the characteristics of the feedstock processed. Biodiversity, with regard to the yeast strain, found in industrial fermentors, is an inexhaustible source used to obtain a microorganism with specific characteristics which may be used in other segments of the bioprocess industry, generating products with higher aggregate value than ethanol.  Some yeast strains with special characteristics, isolated from fermentation processes, have been used in unconventional alcoholic fermentation processes. One example of this type of application is the ethanol obtainment process, which makes used of autoimobilized strains and allows the operation without the separation unit. In this process, it is possible to keep yeast cells "stuck" in the fermentor for their ability to flocculate. This ability leads to the formation of high-density pellets, which are dragged by the must flow, thus originating a stable bed through which the must percolates. In this percolation, sugar is turned into ethanol. This fermented must leaving the fermentor and containing ethanol is sent directly to distillation. This process, in spite of some limitations, has proven to be a viable alternative for ethanol production in small-scale units, where the use of centrifuges may turn their installations unviable. This type of process will have great value to meet the demand in countries with no tradition in ethanol production and, therefore, with no infrastructure of equipment and maintenance. Yeast strains isolated from the Brazilian sugar and alcohol environment are used in a process of this kind, described in patent PCT/BR2009/000030. This process uses this strain for its ability of flocculate and forming pellets. This is just one of the applications for yeasts originated in the biodiversity of the

Brazilian sugar and alcohol industry. Although this text is about yeast strains, a reservation is required concerning the biodiversity of bacteria also inhabiting the sugar and alcohol environment. Many works have approached this topic, but always considering these microorganisms as unwanted in the process, since the presence of bacteria is always associated with some type of problem, whether regarding yield or operations. Although these bacteria are responsible for many problems in the industry, there is another approach which considers these bacteria as sources for the obtainment of new molecules with differentiated properties. Some gums, with unique properties were obtained by isolating the *Leuconostoc* strains, which naturally inhabit Brazilian sugarcane fields (Vieira, 2005, Vieira , 2009).

The study of this artificial ecosystem, the agroindustrial environment, not only leads to the understanding of the population dynamics of yeast strains inhabiting fermentation tanks, but also increases the possibility of obtaining microorganisms with specific characteristics that allow the achievement of bioproducts not yet known. The discovery of these biomolecules may benefit the most distinct industrial segments, including the pharmaceutical, food, petroleum and cosmetics industries, among others.

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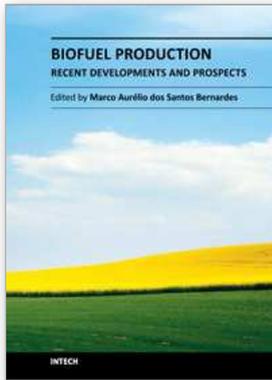
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Edited by Dr. Marco Aurelio Dos Santos Bernardes

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