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The benefits of applied research: 37 years of discoveries, adaptations and solutions

Erfolge angewandter Forschung: 37 Jahre Entdeckungen, Anpassungen und Lösungen

The authors’ work started in fermentation in 1977 and in the 1980’s into sugar production and cane quality. Statistical analysis was a key factor for the success of improving yield in ethanol and sugar production as well as cane quality. Adaption of methods for industrial laboratories also was very important in relation to yield and in reduction of sugar losses in the factory. Methodologies to measure sugar losses occurring through degradation in the factory (evaporation) using ion chromatography and dry substance content with a digital density meter were adapted. The fermentation yield improved from 75% in 1977 to 92% in 2014, which was possible by adapting methods for live bacterial counting within 20 min, and by controlling contamination using antimicrobial products through research in the laboratory and the industry. Since 1990 yeasts for industrial fermentation were selected by karyotyping analysis of the nuclear chromosomes and in the last seven years based on mitochondrial DNA. The last technique made the “Process Driven Selection” possible, i.e. one or several yeast strains which fit each distillery. Floc formation in carbonated beverages is not only due to the Indicator Value (discovery by SPRI research group) but also to aconitic acid and calcium under Brazilian conditions.

Key words: ethanol and sugar production, fermentation, sugar losses, yeast strains, floc formation, applied research

1 Introduction

With the petroleum crises in the 1970’s, Brazil started a program to reduce oil imports and dependency on oil in the energy mix. The PRÓ-ALCOOL program started in 1975 with the aim of increasing the volume of ethanol produced by giving financial benefits to producers and lowering the tax for consumers. Before ethanol was mainly produced by fermentation of diluted molasses with yeast recycle using French technology brought to Brazil at the beginning of the 20th century.


Schlagwörter: Ethanol- und Zuckererzeugung, Fermentation, Zuckerverluste, Hefestämme, Floc-Bildung, angewandte Forschung

Until 1977, fermentation yield was between 70 to 75% and batch fermentation time was around 16 to 20 hours. Ethanol concentration in the fermenters was no higher than 7% (v/v), which means at least 16 L of vinasse per litre of ethanol. The fermenters which were no bigger than 100,000 L were

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increased to 200,000, 400,000, 500,000 L and 1 mn L. Today it is usual to find tanks with a capacity of 3.5 mn L.

With the increases in production, innovations were needed. In this way, Fermentec started to work first on the fermentation process in 1977 and then on sugar production and monitoring of cane quality in the 1980’s. As a consequence of applied research and fieldwork, using yeast recycle fermentation, yield was improved to 92%, the fermentation time reduced to 10 hours and the ethanol concentration increased to 12% (v/v) [1]. In the sugar process it was possible to reduce losses, improve yield and improve cane and sugar quality. These improvements were possible by monitoring process and quality indicators, developing and adapting analytical methodology to monitor the indicators, reducing sugar losses and bacterial contamination and selecting yeast strains more adapted to the industrial environment, as described below.

2 Metrics: linear regression analysis and multiple regression analysis

Statistical analysis was a key factor for the success of improving yields in ethanol and sugar production. Later also it was a key in improving cane quality which impacts the sugar and ethanol recovery.

Over the last 37 years, Fermentec has accumulated data from more than 120 parameters related to cane quality, sugar processing and the ethanol fermentation process and, by applying statistical analysis as simple and multiple regression and correlation, it was possible to identify the main parameters associated with yields, efficiency, losses and quality. As an example in Figure 1 it is possible to observe how the dextran content in cane juice is dependent of the amount of soil brought in with the cane. Figure 2 shows how the fermentation yield is reduced by increased mash acidity due to bacterial contamination. Regarding sugar processing, Figures 3 and 4 shows how mineral impurities (soil that comes with the cane) and dextran can reduce the sugar recovery. Figure 5 shows how the quality

Fig. 2: Ethanol yield versus acidity of fermented mash

Fig. 3: Sugar recovery versus mineral impurities in sugar manufacture

Fig. 4: Sugar recovery according to SJM formula versus dextran content in cane mixed juice (on dry substance)

Fig. 5: Relationship between dextran and soil in cane based on cane quality data from an entire harvest season
of VHP sugar is affected by the amount of dextran that enters the factory with the cane. The results of statistical analysis also give to industrial managers the sense of necessity to improve the process and to provide the right investments to improve efficiency and quality. Besides, knowing the parameters which can affect the process can help to reduce losses and consumption of chemicals such as antibiotics, lime, polymers, sulfite, sulfuric acid and anti-foaming agents [1].

3 Analytical methods and sampling for cane, ethanol and sugar

Adaption of methods for an industrial laboratory also was very important in relation to yield and in the reduction of sugar losses in the factory. The analytical results in a sugar mill and distillery laboratory have two objectives: the first one is to calculate the fermentation yield, the sucrose recovery and also the global industrial efficiency, that is, how much sugar and ethanol are produced in relation to the sugar entering the factory. The second objective is to monitor and manage the performance of the fermentation and sugar process, in order to understand the variations in their performance. By understanding those variations, it is possible to avoid undesirable behavior in the process.

3.1 Sugar determination

Nowadays, sugars can be easily measured by high performance liquid chromatography (HPLC) with refractometric index detector or by high performance ion chromatography (HPIC) with pulsed amperometric detector. However, although a great number of mills have chromatographs in their laboratory in these days, back in 1975 this equipment was a “technical dream”. In this way, Total Reducing Sugars (TRS) in juices, syrup, molasses, mash and fermented mash were measured by the Lane-Eynon titration method [2] from 1975 to 1985. The procedure is based in the reduction of copper (II) ions and since the reaction is not stoichiometric and involves the heating of the solution the precision was very poor. To solve the precision problem, equipment developed at the University of São Paulo (ESALQ) improved the determination performance and reduced the labor intensity by carrying out the reaction in a chamber heated with water vapor [3]. In 1992, Fermentec improved the equipment by adding a redox electrode to determine the titration endpoint. With the electrode a better accuracy and repeatability was obtained [4]. However, this methodology does not measure well the residual sugars at the end of the fermentation, due to the low sugar content in this kind of sample. In this way, in 1978, Fermentec adapted the Nelson-Somogyi method for blood analysis [5] (also based in the reduction of copper(II) ion) for the analysis of mash, fermented mash and industrial waters which has a good accuracy for contents down to 7–10 mg/L [6]. In 2005, Fermentec modified the methodology changing the reagent sodium arsenate to a “greener” reagent, sodium silicate [7]. In respect to chromatography for measuring sugars, between 1975 to 1995 it was used only in research laboratories, not in the industry, but in 1995 some plants started using this equipment. In one analytical run, it is possible to measure sucrose, glucose, fructose, glycerol and mannitol [8]. Glycerol and mannitol, as well as lactic and acetic acid enter the mass balance to help to calculate fermentation yield, and give an indication of possible problems. Today, chromatography equipment is more affordable, and therefore used in sugar mills and distilleries laboratories especially for molasses and deteriorated cane. Such samples contain reducing substances that react with copper ions and interfere in the Lane-Eynon method and the Nelson-Somogyi method, contributing to overestimating the total sugars content.

3.2 Ethanol determination

Ethanol concentration was determined in 1975 by ebulimeter or by chemical oxidation. The ebulimeter has low accuracy. Therefore it is difficult to measure low concentrations of ethanol in the stillage. This low concentration may still represent a big loss due to great volume of the stillage. 0.05% (v/v) ethanol concentration means 0.6% loss of the total ethanol production. The chemical reaction (oxidative) is time-consuming and labor-intensive. In this manner, in 1978, Fermentec adapted a Micro Kjeldahl distillation apparatus to extract the ethanol. After the vapor distillation, the density of the resulting ethanol solution is measured with a density meter and the ethanol concentration is calculated. Compared to gas chromatography, the density meter procedure is more robust and does not need reagents or gases to carry out the analysis. In this way the cost per analysis is lower. By better measuring the loss of ethanol in vinasse or stillage it was possible to improve the distillation process and reduce losses. In Figure 6 is presented how losses of ethanol decreased with process monitoring with the density meter procedure after 1978.
3.3 Glycerol determination

The determination of glycerol is important in order to calculate the fermentation yield by mass balance, and to monitor the yeast behavior. A high glycerol formation can mean that the yeast is suffering a stress or it is multiplying vigorously. From 1975 to 1985 it was not possible measure glycerol on site, but only in research laboratories. In 1985 the plants started to measure glycerol by chemical reaction [9]. In 1995, Fermentec adapted an enzymatic reaction kit [10] used for triglycerides determination in serum or plasma for glycerol determination in mash. The decision to employ an assay kit used in clinical analysis was taken to reduce the analysis cost, since the triglycerides assay kit for clinical analysis was much less expensive than the cost of enzymes and reagents purchased separately. Nowadays, with the popularity of HPLC and HPIC in the mills and distilleries, glycerol is measured also by chromatography.

3.4 Total acidity and nitrogen

The increase of acidity during fermentation indicates the increase of bacterial contamination. The analysis of this parameter helps the process control and management. In the future, this analysis will be substituted by HPLC and HPIC for the determination of specific of organic acids produced by bacteria such as lactic and acetic acid. Figure 2 shows how the fermentation yield is influenced by the acidity. Nitrogen is an important element that needs to be measured. From 1975 to 1985, total N was measured, but no correlation was found with fermentation time, yield etc. Then, in 1985 Fermentec adapted an ammonia-nitrogen method [11] to measure it in the mash, and fermented liquor. Good correlations were found between fermentation time and ammonia-nitrogen in the mash, and also in the yeast propagation process.

In addition to \( \text{NH}_4^+ \), amino acids and small peptides are present in the mash which are also metabolized by the yeast. Therefore, Fermentec adapted a method that uses formaldehyde [12] to measure the assimilable nitrogen. Here all nitrogen sources that are available and are metabolized by yeast (\( \text{NH}_4^+ \) and free amino acids) are measured. This analytical method gave excellent correlation with the parameters mentioned above (fermentation time and yeast propagation process). In addition, in a sugar factory, a good correlation (\( r = 0.708 \)) between the content of free amino acids and sugar color was observed (Fig. 7) [13].

3.5 Near Infrared Spectroscopy (NIR)

Near Infrared Spectroscopy has been used in Brazil by Fermentec in mills and distilleries since 1993, being one of the first in the world to apply this technique in sugar and ethanol factories. Many mills and distilleries use it these days. However, the price and problem of calibration have impaired a broad usage of this technique. NIR has been used to measure the following compounds and solutions:
- Cane: Determination of total sugar directly in shredded cane for growers payment.
- Mash: Content of total sugar as invert (also sucrose, fructose and glucose) and solids.
- Fermented mash: Content of ethanol, glycerol, residual sugars (glucose, fructose or sucrose), and yeast fraction (v/v).
- Treated yeast: Content of ethanol, glycerol, and yeast fraction (v/v).
- Centrifuge: yeast cream concentration and yeast lost in centrifuged beer.

Although the NIR calibration for a sugarcane mill and distillery is labor intensive and requires many samples due to the large sample variation which occurs during the harvest season, the use of this technology is promising because many parameters may be measured in less than one minute. However, it should be stressed that success in NIR application requires use of adequate standards and trained people.

The Brazilian mill Vale do Rosario was the first one in the world to use a NIR spectrometer to measure sugar directly in cane for cane growers’ payment in 1993. Figure 8 shows a correlation between total reducing sugars (glucose + fructose + sucrose) measured directly in shredded cane by NIR spectrometer and total reducing sugar measured in shredded cane juice by conventional analysis in the laboratory. In this case, since...
the analysis by NIR is carried out in a solid, particle size is a very important parameter. Therefore shredded cane was reprocessed by a MES™ (Maintenance Engineering Services CC) cane shredder [14] in order to increase the amount of open cells and standardize the particle size without losing humidity. Regarding fermentation, Figure 9 shows a correlation obtained between the ethanol content in fermented mash (beer) measured in the distillery laboratory using a density meter and the ethanol content measured directly by NIR system. As can be seen in the Figure, the correlation between the results was very good, indicating the availability of the technique to control the fermentation process.

### 3.6 Microbiological analysis

Fermentation is a living process, so the monitoring of the yeast and its contamination is essential for successful ethanol production. From 1975 to 1985, Fermentec used the traditional coloring method [15] for measurement of the viability of yeast. However, in several plants the color did not show up, and it was difficult to know if the yeast was alive or not. In 1995 Fermentec introduced another known method that used the erythrosine [16]. It is much better and due to the strong red color, fewer problems have been observed. Six years ago, Fermentec started research to find a method that not only shows the viability but also the vitality of the yeast cell. By using the phase contrast microscopy [27], it was possible to show the excellent correlation with the erythrosine methodology in viability [17].

The yeast has to compete with bacteria, and to count living bacteria it takes two to three days by plating counting. To speed up the analysis, Fermentec adapted a method that was used for counting bacteria in milk to count living bacteria in the fermented mash and related samples with a limit of detection of 105 bact/mL [18]. Bacteria, mainly Lactobacillus and Bacillus, affect fermentation ethanol yield by inhibiting and deviating the yeast products. The production of ethanol decreases and glycerol increases. In Figure 10 shows how the number of bacteria affect fermentation yield. In this way, Fermentec developed a rapid test (6 hours) to evaluate the sensitivity of bacterial strains to antimicrobial compounds, so the antimicrobial costs are minimized, once only the most effective one is applied [19].

### 3.7 Industrial sampling

The increasingly modern laboratory analytical techniques minimize the analytical uncertainty, but to obtain reliable data it is essential that the sample used in this analysis is representative, that is, the sample shows physicochemical characteristics as similar as possible to the characteristics found in the process [20]. A solution to the problem of representativeness is the collection of a larger number of samples. In this way, Fermentec introduced the automation of the sampling process with sample conservation. By composing the sample dur-
ing a period, the measurements were much more consistent, and deviations by deficiencies accounting for sampling were reduced considerably [21].

After 1995, the frequency of the sampling made by continuous automatic samplers started to be proportional to the mill flowrate instead of elapsed time. This helped to represent even better the process characteristics and to quantify the absolute amount the sugar lost. Today samplers are equipped with refrigerator in order to preserve the sample and an automatic cleaning system to avoid bacterial contamination.

Considering shredded cane samples for core laboratory analyses, in order to improve sample homogeneity of solids particles, Fermentec introduced the use a small modified concrete mixer to homogenize the sample before analyses. Using the mixer improves the repeatability and reliability of results.

3.8 Determination of the volume of fermented mash (batch process)

In order to quantify ethanol yield and fermentation efficiency, sugar balance and losses, it is very important to measure the volume of mash and fermented mash in the fermentation process. Because of variations in process conditions which occur, especially in density (due to sugar content variation), the use of a flowmeter does not have the required certainty to calculate the yield with an acceptable degree of accuracy. Therefore, in batch fermentation processes the volume involved in fermentation is measured directly in the fermentation tank. Before 1995, an accurate volume measure was only possible visually with a lateral level pipe with a calibrated graduation. After 1995 in a partnership with SMAR, Fermentec introduced the Level Meter for Guided Waves (TDR Technology). Using this technology, it is possible to measure the height of a liquid column automatically and then calculate the fermentation volume with appropriate certainty, without the necessity of human interference.

4 Results of applied research and solution of problems in the ethanol and sugar industries

4.1 Ethanol production and yeast selection

For yeast monitoring and selection, the karyotyping of nuclear chromosomes is used. In this way it is possible to know if the yeast which are in the fermenters are *S. cerevisiae* and among the *S. cerevisiae* it is the strain, with which the fermentation was started [22]. By this analysis, it was possible to verify that the traditional baker’s yeast could not survive the stressful conditions of industrial fermentation with cell reuse. Moreover, monitoring the strain in the industrial process during the seasons it was possible to screen wild strains able to survive and dominate the fermentation which have good fermentation characteristics such as: high ethanol yield, low glycerol formation, low foam formation, high viability etc. This screening has been a continuous process over the past 25 years, and today, among others, Fermentec has commercially available three-selected yeasts responsible for 70% of all ethanol produced in Brazil: PE-2, CAT-1 and FT858 [23].

The monitoring of yeast in the process led to important discoveries about the ability of yeast to dominate and stay in a particular distillery. In this way, it was possible to isolate and select a particular strain more adapted for a particular production plant, as a “customized” yeast. Since 2007, Fermentec has introduced customized yeast and in 2014 the production of ethanol by these yeasts was over 3 bn L. The superiority of these yeasts is unquestionable, since 100% of persistence in the fermenters was obtained, with an average of 89% of dominance, which means a greater stability of the fermentation process. As the yeast is isolated and reintroduced into the same plant, it is more adapted to the particular process, so it has, consequently, more persistence and dominance. In the 2014 season, 14 “customized” yeasts were re-introduced in plants to begin the fermentation harvest season.

It is sometimes difficult to know by karyotyping if some yeasts are the same ones, since 2007 Fermentec has been using another technique, analysis of mitochondrial DNA, which shows whether the yeast is the same or not, or if it is a relative.

4.2 Sugar degradation in the factory

Monitoring and control of the points of sugar losses are extremely important in view of the impact they have on the financial gains [24]. As an example, considering a mid-sized plant with an annual grinding of 2,000,000 t of sugarcane, whose sucrose content in the raw material is 15.5% on average, a loss of 1% sugar during the process means the loss of 59,000 bags of sugar or 1.85 mn L of alcohol per year [25]. One of the main points of sugar losses are the residual waters and cane cleaning water. However, a lot of sugar can be destroyed in the evaporation process. Fermentec has been studying the hydrolysis of sucrose and degradation of sugar for many years with international collaboration and concluded [24]

- Losses in pre-evaporators are higher than in multiple effects.
- Reducing sugars (glucose and fructose) accelerate the hydrolysis of sucrose.
- Scaling in pre-evaporators and multiple effects increase the losses by degradation.

It was observed that increasing efforts to remove scaling, reduce sugar degradation, as can be seen in Table 1, when sugar content in evaporators were monitored before and after cleaning, respectively.

4.3 Floc formation in non-alcoholic beverage

The presence of floc in soft drinks is a concern for producers of carbonated beverages and sugar quality may be one source of this problem. Fermentec, during a sugar quality research, observed that the causes of these flocs are mainly related to aconitic acid, calcium and Indicator Value (IV). Usually these parameters are not evaluated during routine quality control of white crystal sugar, but were very helpful to distinguish samples of sugars classified as low, medium and high potential to form flocs in soft drinks. It was observed that the probability of soft drinks to form flocs increases when a sugar used in...
the composition has aconitic acid values greater than 70 mg/kg, Indicator Value over 5 and calcium contents greater than 20 mg/kg. According to the multivariate regression analysis, these three factors are related to 59% (p < 0.001) of the variance observed in the potential of sugars to form floc.

It should be noted that these indicators are directly related to the processing of sugarcane and to the cane quality. The green cane harvested mechanically has many plant impurities (leaves and tops) that affects the industrial process and the recovery of sugar. For this reason, due to the increase of mechanically-harvested sugarcane and consequent increase in the raw material impurities, Fermentec recommends that these new sugar quality indicators are introduced for quality analysis to predict the potential of sugar to form flocs in soft drinks.

### 4.4 Impact on profit in 33 distilleries

A study with the objective of measuring the economic gains provided by increased efficiency of industrial alcohol distilleries that were accompanied by Fermentec was conducted by University of São Paulo, Brazil [26].

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<table>
<thead>
<tr>
<th>Sample</th>
<th>pH value</th>
<th>Glucose in g/100 g rds</th>
<th>Fructose in g/100 g rds</th>
<th>Sucrose in g/100 g rds</th>
<th>Monosaccharides in g/100 g rds</th>
<th>Total sugar in g/100 g rds</th>
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<tbody>
<tr>
<td>Before cleaning</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
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<td>86.56</td>
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<td>84.23</td>
<td>9.02</td>
<td>97.68</td>
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<tr>
<td>Juice Pre-evaporator #2</td>
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<td>2.92</td>
<td>2.46</td>
<td>83.64</td>
<td>5.37</td>
<td>93.41</td>
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<td></td>
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<tr>
<td>Clarified Juice</td>
<td>6.63</td>
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<td>82.99</td>
<td>4.89</td>
<td>92.24</td>
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</table>
Information from clients of Fermentec between 1977 and 2007 were analyzed before and after the plants received guidance in order to measure the benefits of applied research. The study observed a positive correlation with the distillery efficiency and the time that those plants were supported by Fermentec expertise. Considering the period under review, the applied research in the last three decades in 33 plants showed a net profit of around USD3.5bn, only from increasing the ethanol efficiency production.

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