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A modified indirect mathematical model for evaluation of ethanol production efficiency in industrial-scale continuous fermentation processes

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Abstract:	To obtain a robust method to calculate fermentation efficiency in a continuous ethanol production process we developed a mathematical method based on the analysis of metabolic by-product formation in order to indirectly calculate the process efficiency. This method is in contrast to the traditional way of calculating ethanol fermentation efficiency, where final ethanol concentration and total glucose consumption during fermentation is expressed as a percentage (%) of the theoretical conversion yield. When comparing both calculation methods at industrial-scale and in sensitivity studies, it was observed that the indirect method was more robust and gave slightly higher fermentation efficiency values, although overall fermentation efficiency of the industrial process was found to be low (~75%). The low fermentation efficiency obtained shows an urgent need for industrial process optimization where the indirect calculation methodology will be an important tool to determine process losses.
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Las Talitas, Tucumán, Argentina, January 13th, 2016

To the Editorial Board of *Journal of Industrial Microbiology and Biotechnology*

Dear Editor,

Please find enclosed the manuscript entitled "**A modified indirect mathematical model for evaluation of ethanol production efficiency in industrial-scale continuous fermentation processes**" for evaluation in the *Journal of Industrial Microbiology and Biotechnology*. This manuscript has not been submitted for publication elsewhere and does not present any kind of conflict of interests.

This manuscript represents an original work on the development of an accurate and robust method to calculate fermentation efficiency in an industrial-scale continuous ethanol production process. This is to our knowledge the first comparison or validation of different mathematical methods to calculate fermentation efficiency in a continuous cascade process on an industrial scale. By employing the indirect calculation method presented in this manuscript it is possible to determine the stoichiometry of metabolites of the industrial fermentation process, which enables to rapidly identify, correct and solve a punctual problem and to recover high ethanol production yield. The low overall fermentation efficiency found for the ethanol industrial process in Tucumán, highlights the need for a robust calculation methodology as an important tool in order to rapidly and efficiently optimize the ethanol production process.

The corresponding author signs this cover letter on behalf of all authors.

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Full title: A modified indirect mathematical model for evaluation of ethanol production efficiency in industrial-scale continuous fermentation processes

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Short title: Evaluation of alcoholic fermentation efficiency by an indirect method

ABSTRACT

To obtain a robust method to calculate fermentation efficiency in a continuous ethanol production process we developed a mathematical method based on the analysis of metabolic by-product formation in order to indirectly calculate the process efficiency. This method is in contrast to the traditional way of calculating ethanol fermentation efficiency, where final ethanol concentration and total glucose consumption during fermentation is expressed as a percentage (%) of the theoretical conversion yield.

When comparing both calculation methods at industrial-scale and in sensitivity studies, it was observed that the indirect method was more robust and gave slightly higher fermentation efficiency values, although overall fermentation efficiency of the industrial process was found to be low (~75%). The low fermentation efficiency obtained shows an urgent need for industrial process optimization where the indirect calculation methodology will be an important tool to determine process losses.

KEY WORDS: fermentation efficiency, bioethanol, mathematical model, indirect calculation method, continuous fermentation process.

NOMENCLATURE

Direct method

Ed Fermentation efficiency percentage by direct method [%]
Yr Real ethanol yield [L/kg]
Yt Theoretical ethanol yield = 0.6475 [L ethanol/kg sugars]
P Ethanol produced [L/h]
S Fed Sugars [kg/h]
f°_{W} Wine flow [m3/h]
f°_{M} Molasses flow [m3/h]
f°_{J} Cane juice flow [m3/h]
p_W Wine ethanol concentration [mL/100mL]
ts_M Molasses total reducing sugars [g/100g]
us_M Molasses unfermentable sugars [g/100g]
ts_J Cane juice total reducing sugars [g/100g]
ρ_M Molasses density [kg/L]
ρ_J Cane juice density [kg/L]

Indirect method

Ei Fermentation efficiency percentage by indirect method [%]
Yt Theoretical ethanol yield = 0.511 [kg ethanol/kg sugars]
Pg Ethanol mass flow generated [kg/h]
Xg Cells mass flow generated [kg/h]
Gg Glycerol mass flow generated [kg/h]
Ag Acid mass flow generated [kg/h]
Ro Residual sugars mass flow [kg/h]
P'_{FM} Fermented must ethanol mass flow [kg/h]
P'_{YC} Treated Yeast cream ethanol mass flow [kg/h]
P'_{A} Alimentation must ethanol mass flow [kg/h]
X'_{FM} Fermented must cells mass flow [kg/h]
X'_{YC} Treated Yeast cream cells mass flow [kg/h]
X'_{A} Alimentation must cells mass flow [kg/h]
G'_{FM} Fermented must glycerol mass flow [kg/h]
G'_{YC} Treated Yeast cream glycerol mass flow [kg/h]
G'_{A} Alimentation must glycerol mass flow [kg/h]
A'_{FM} Fermented must acid mass flow [kg/h]
A'_{YC} Treated Yeast cream acid mass flow [kg/h]
A'_{A} Alimentation must acid mass flow [kg/h]
R'_{FM} Fermented must residual sugars mass flow [kg/h]
f°_{FM} Fermented must flow [m3/h]
f°_{YC} Treated Yeast cream flow [m3/h]
f°_{A} Alimentation must flow [m3/h]
f°_{AW} Alimentation dilution water flow [m3/h]
f°_{YCW} Yeast cream dilution water flow [m3/h]
p_{FM} Fermented must ethanol concentration [mL/100mL]
x_{FM} Fermented must cells concentration [mL/100mL]
g_{FM} Fermented must glycerol concentration [g/100mL]
a_{FM} Fermented must acids concentration [mg/100mL]
r_{FM} Fermented must residual sugars concentration [g/100mL]
p_{YC} Treated Yeast cream ethanol concentration [mL/100mL]
x_{YC} Treated Yeast cream cells concentration [mL/100mL]
g_{YC} Treated Yeast cream glycerol concentration [g/100mL]
a_{YC} Treated Yeast cream acid concentration [mg/100mL]
a_A Alimentation must acid concentration [mg/100mL]
Ethanol density 20ºC = 0.7893 [kg/L]
] Relationship between dry and wet yeast mass 0.3 [kg dry yeast/ L wet yeast]

INTRODUCTION

The inevitable depletion of the world's fossil energy supply, has generated an urgent and imminent global need to find alternative renewable sources of energy and fuels [1]. One of the most important candidates to replace gasoline and natural gas as transportation fuel is ethanol, which is now considered a profitable commodity by its increasing use as an additive and/or fuel for car gasoline engines [2].

Ethanol may be obtained from different sugar-containing substrates, but in order to obtain an economically competitive production it is important to keep substrate costs low [3]. Currently, global ethanol supply is almost exclusively produced from sugarcane and corn feedstocks [4] where the ethanol production process is based on the fermentative activity of brewer´s yeast (*Saccharomyces cerevisiae*) [5]. The fermentation process is one of the most critical steps in a distillery, as it is here that the yeast

converts sugars to ethanol. It is also in this step, that contaminating microorganisms have the opportunity to divert the ethanol fermenting process producing other metabolic products such as lactic acid, glycerol and acetic acid among others [6].

Over the last 30 years the brewing and distilling industry have developed new and more efficient fermenting systems including rapid batch fermentation using cylindro-conical or sloping bottom fermentors and continuous fermentation using a cascade of fermentors [6] The traditional ethanol fermentation system uses suspended yeast cells in a single bioreactor filled with sugar substrate, where the total reactor volume (‘‘batch’’) is gradually fermented and subsequently removed from the reactor. By contrast, continuous fermentation system has a steady input of medium into the fermentor and a corresponding uninterrupted output of fermented product is taken out of the system. In its simplest one-reactor form, the continuous fermentor operates at steady state with a volume content entering the system equal to the finished product that is taken out of the system. Alternatively, a cascade of interconnected separate fermentors can be used to avoid a direct flow of unfermented medium into the near-finished product [7]. The most successful continuous fermentation system used in distilling is the cascade system where most modern cascade plants operates with five fermentors and a pre-fermentor [6], where the yeast can be centrifuged, washed and reused.

At the moment both, batch and multistage continuous processes for industrial production of bioethanol [8], are used. One disadvantage of batch ethanol fermentation is the significant downtime (cleaning, sanitizing and filling) between runs, which represents an important loss in effective production time leading to less profitability. The continuous fermentation process on the other hand provides several advantages over the batch fermentation: where optimized process conditions for maximal productivity, long-term continuous production, higher volumetric productivity, reduced labor costs once steady state is reached, reduced vessel down-time for cleaning, filling and sanitizing and easier process control and operation are the most important ones. However, successful and efficient application of continuous fermentation is only possible if the challenge of yeast cell metabolism dynamics and microbe contaminations can be overcome and controlled [9].

The yield of ethanol is the main parameter to be evaluated in the industrial process of alcohol fermentation, but fermentation optimization is a complex procedure because of the many parameters that can affect the final alcohol content. It is therefore important to present a reasonable level of automation, and perform frequent analytical measures during the batch cycle to be able to control the process [6][10].

Traditionally, the ethanol conversion yield is calculated by the ratio between the final ethanol concentration and the sugars consumed (the difference between initial and residual sugar concentrations) and is expressed as a percentage (%) of the theoretical conversion yield, which is 0.511 g of ethanol/g of glucose [11] [12]. Additionally to the traditional calculation form, ethanol yield can also be calculated by indirect calculation methodology based on the different non-ethanol by-products formed during the fermentation process. This model quantifies the losses generated by each one of the metabolic by-products, such as carbon dioxide, organic acids and glycerol, formed during the process as a result of the deviation of fermentable sugars that was not transformed into ethanol. This method of indirectly calculating ethanol yield, called "the method of losses", was first presented by Finguerut *et al.* (1985) and later modified and applied at laboratory scale [13]. In the latter study, it was shown that results obtained from this indirect calculation method did not differ significantly from the direct method and that it was more robust since it showed less variability between experiments. However, to date there are no studies published on production of bioethanol in which both methods have been compared or validated when calculating the efficiency of fermentation in a continuous flow operation on an industrial scale.

Therefore, the aim of this study was to develop a mathematical model capable of estimating efficiency of yeast fermentation by quantifying secondary metabolites during ethanol production in a continuous cascade system. In addition, we also tested the validity of the model using experimental data from a local distillery and finally we analyzed the robustness of the model through a sensitivity study.

MATERIALS AND METHODS

1 Mathematical models

1.1 Fermentation efficiency by traditional methodology (Direct Method - DM)

Fermentation efficiency is calculated, using the direct method, as the true ethanol yield divided by the theoretical yield multiplied by 100 [14][12]:

$$E_d = \frac{Yr}{Yt} * 100 = \frac{P}{S * 0.6475} * 100$$

Equation 1

Where: E_d: Fermentation efficiency percentage by direct method [%], Yr: Real ethanol yield [L/kg], Yt: Theoretical ethanol yield = 0.6475 [L ethanol/kg sugar], P: Ethanol produced [L/h] and S: Fed Sugars [kg/h].

In order to calculate the efficiency of ethanol production using the direct method for a continuous cascade system containing "n" fermentors, the whole fermentation process was considered as a "black box", where inflows considered were molasses, cane juice, alimentation dilution water and yeast cream dilution water and the output current, was centrifuged wine (**Fig 1**).

Fig 1.tiff: Schematic model of the "black box" (dotted black line) of the continuous ethanol fermentation system which was used for the mathematical calculation of the ethanol production efficiency using the direct model.

In order to calculate the efficiency of alcoholic fermentation process using the black box model presented in **Fig 1**, the terms involved in the above mentioned formula were defined as follows:

$$P = (f^{\circ}_{W} * 1000) * \frac{p_W}{100}$$

Equation 2

$$S = \left((f^{\circ}_{M} * 1000) * \rho_M * \left(\frac{ts_M}{100} - \frac{us_M}{100}\right)\right) + \left(\left(f^{\circ}_{J} * 1000\right) * \rho_J * \frac{ts_J}{100}\right)$$

Equation 3

Where: P Ethanol produced [L/h], S Fed Sugars [kg/h], f°_{W} Wine flow [m3/h], p_W ethanol concentration in Wine [mL/100mL], f°_{M} Molasses flow [m3/h], ρ_M Molasses density [kg/L], ts_M total reducing sugars in Molasses [g/100g], us_M unfermentable sugars in Molasses [g/100g], f°_{J} Cane juice flow [m3/h], ρ_J Cane juice density [kg/L] and ts_J total reducing sugars in Cane juice [g/100g].

The unfermentable sugars from cane juice were considered to be negligible, and were therefore not included in the second term of Equation 3.

1.2 Fermentation efficiency using a by-products methodology (Indirect Method - IM)

There are two major reasons by which fermentation yield may decrease: firstly, the existence of unfermented sugar and secondly, the formation of other metabolites different than ethanol by the yeast and/or other microbial contaminants [13]. Therefore, in an attempt to better describe the fermentation efficiency Finguerut *et al.*, (1985) proposed a stoichiometric mass balance for the fermentation process as described below:

$$\text{sugar} + \text{nutrient} = \text{ethanol} + CO_2 + \text{yeasts} + \text{glycerol} + \text{acids} + \text{residuals sugars} + \text{other products}$$

If we consider, in the equation proposed, that the mass of the "nutrients" used (amino acids, ammonium salts, phosphorus and other salts) is equal to the mass of all of the "other products" (acetaldehyde, esters, fusel alcohols, acetone, etc.), and where the mass of CO_2 is equal to the mass of ethanol produced without generating a significant error the following mathematical equation for calculating the efficiency of ethanol fermentation was proposed [13]:

$$E_i = \frac{100}{0.511 * \left(2 + \frac{X_g}{P_g} + \frac{G_g}{P_g} + \frac{A_g}{P_g} + \frac{R_o}{P_g}\right)}$$

Equation 4

Where: Ei: Fermentation efficiency percentage by indirect method [%] Yt: Theoretical ethanol yield = 0.511 [kg ethanol/kg sugar], P_g: Ethanol mass flow generated [kg/h], X_g: Cells mass flow generated [kg/h], G_g: Glycerol mass flow generated [kg/h], A_g: Acid mass flow generated [kg/h] and R_o: Residual sugars mass flow [kg/h].

In this study we propose a simplified model of equation 4, shown in **Fig 2**, for calculating ethanol production efficiency in a continuous cascade fermentation system of "n" fermentors. This model considers the whole fermentation system as a "black box" that comprises all the fermentation tanks. Input flow is the alimentation must and treated yeast cream, whereas the output flow is the fermented must prior to centrifugation.

Fig 2.tiff: Schematic model of the black box (dotted black line) considered for the continuous ethanol fermentation system used for the mathematical model of ethanol efficiency calculation using the indirect calculation method.

Using this model, mass balances were calculated by determining cell biomass, glycerol concentration, acid concentrations, residual sugars and ethanol concentration. The relations expressed in Equation 4 were defined from the following general balance:

$$\text{input} + \text{generation} = \text{output}$$

1.2.1 Ethanol mass flow generated (P_g)

The balance for ethanol mass of the continuous fermentation process can be written as follows:

$$P_{generated} = P_g = P'_{FM} - P'_{YC} - P'_A$$

Assuming that mass P'_A is negligible, and substituting in the equation above:

$$\mathbf{P_g} = \left((f^{\circ}_{FM} * 1000) * \frac{p_{FM}}{100} * 0.7893\right) - \left((f^{\circ}_{YC} * 1000) * \frac{p_{YC}}{100} * 0.7893\right)$$

Equation 5

Where, 0.7893 [Kg/L] is the ethanol density at 20°C, P'_{FM}: ethanol mass in Fermented must flow [kg/h], P'_{YC}: ethanol mass in Treated Yeast cream flow [kg/h], P'_A: ethanol mass Alimentation must flow [kg/h], f°_{FM}: Fermented must flow [m3/h], p_{FM}: ethanol concentration in Fermented must [mL/100mL], f°_{YC}: Treated Yeast cream flow [m3/h] and p_{YC}: ethanol concentration in Treated Yeast cream [mL/100mL].

1.2.2 Cell mass flow generated (X_g)

The balance for cell mass of the continuous ethanol fermentation process can be written as follows:

$$X_{generated} = X_g = X'_{FM} - X'_{YC} - X'_A$$

Assuming that mass X'_A is negligible, and substituting in the equation above:

$$\mathbf{X_g} = \left((f^{\circ}_{FM} * 1000) * \frac{x_{FM}}{100} * 0.3\right) - \left((f^{\circ}_{YC} * 1000) * \frac{x_{YC}}{100} * 0.3\right)$$

Equation 6

Where, 0.3 is a factor that considers the relation between the dry and the fresh weights of yeast, X'_{FM}: cells mass in Fermented must flow [kg/h], X'_{YC}: cells mass in Treated Yeast cream flow [kg/h], X'_A: cells mass in Alimentation must flow [kg/h], f°_{FM}: Fermented must flow [m3/h], x_{FM}: cells concentration in Fermented must [mL/100mL], f°_{YC}: Treated Yeast cream flow [m3/h] and x_{YC}: cells concentration in Treated Yeast cream [mL/100mL].

1.2.3 Glycerol mass flow generated (G_g)

The balance for glycerol mass of the continuous fermentation process can be written as follows:

$$G_{generated} = G_g = G'_{FM} - G'_{YC} - G'_A$$

Assuming that mass G'_A is negligible, and substituting in the equation above:

$$\boldsymbol{G_g} = \left((f^{\circ}_{FM} * 1000) * \frac{g_{FM}}{100}\right) - \left((f^{\circ}_{YC} * 1000) * \frac{g_{YC}}{100}\right)$$

Equation 7

Where: G'_{FM}: glycerol mass in Fermented must flow [kg/h], G'_{YC}: glycerol mass in Treated Yeast cream flow [kg/h], G'_A: glycerol mass in Alimentation must flow [kg/h], f°_{FM}: Fermented must flow [m3/h], g_{FM}: glycerol concentration in Fermented must [g/100mL], f°_{YC}: Treated Yeast cream flow [m3/h] and g_{YC}: glycerol concentration in Treated Yeast cream [g/100mL].

1.2.4 Acid mass flow generated (A_g)

The balance for acid mass of the continuous fermentation process can be written as follows:

$$A_{generated} = A_g = A'_{FM} - A'_{YC} - A'_A$$

By substituting in the equation above:

$$\boldsymbol{A_g} = \left((f^{\circ}_{FM} * 1000) * \frac{a_{FM}}{100 * 1000}\right) - \left((f^{\circ}_{YC} * 1000) * \frac{a_{YC}}{100 * 1000}\right) - \left((f^{\circ}_{A} * 1000) * \frac{a_A}{100 * 1000}\right)$$

Equation 8

Where: A'_{FM}: acid mass in Fermented must flow [kg/h], A'_{YC}: acid mass in Treated Yeast cream flow [kg/h], A'_A: acid mass in Alimentation must flow [kg/h], f°_{FM}: Fermented must flow [m3/h], a_{FM}: acid concentration in Fermented must [mg/100mL], f°_{YC}: Treated Yeast cream flow [m3/h], a_{YC}: acid concentration in Treated Yeast cream [mg/100mL], f°_{A}: Alimentation must flow [m3/h] and a_A: acid concentration in Alimentation must [mg/100mL].

1.2.5 Residuals Sugars mass flow (R_o)

In this particular case the employed mass balance was:

$$\text{input} - \text{consumption} = \text{output}$$

Residual sugar mass for the continuous fermentation process can be calculated as follows:

$$R_{output} = R_o = R'_{FM}$$

By substituting in the equation above:

$$\mathbf{R_o} = \left((f^{\circ}_{FM} * 1000) * \frac{r_{FM}}{100} \right)$$

Equation 9

Where: R'_{FM}: residual sugars mass in Fermented must flow [kg/h], f°_{FM}: Fermented must flow [m3/h] and r_{FM}: residual sugars concentration in Fermented must [g/100mL].

2 Practical Application of the proposed Indirect Mathematical Model at Industrial Scale

2.1 Sampling

Evaluation of the effectiveness of the modified indirect method was conducted using experimental data collected from a local ethanol industry in the Province of Tucumán at two different time points, September 2013 and September 2014. Samples (500mL) were collected in quadruplicate in sterilized plastic bottles at the following stages of the ethanol production process: sugarcane juice, molasses, alimentation must, final fermentation tank, wine and yeast cream (**Fig 1** and **Fig 2**). The temperature of all samples was measured on site with an infrared thermometer (RAYNGER ST-4, RAYTEK) and samples were then transported on ice to the laboratory and stored at −20°C until processed.

The ultrasonic clamp-on flow measurement technology was used to determine the flow values of input and output streams (FLUXUS F601, FLEXIM). Flow values read in online flowmeter were molasses, juice, alimentation dilution water and yeast cream dilution water (added to centrifuge output and input pre-fermentor). From these values, we could calculate flows that could not be measured directly as follows:

$$f^{\circ}_{A} = f^{\circ}_{M} + f^{\circ}_{J} + f^{\circ}_{AW}$$

$$f^{\circ}_{FM} = f^{\circ}_{A} + f^{\circ}_{YC}$$

$$f^{\circ}_{W} = f^{\circ}_{A} + f^{\circ}_{YCW}$$

Where: f°_{A}: Alimentation must flow [m3/h], f°_{J}: Cane juice flow [m3/h], f°_{M}: Molasses flow [m3/h], f°_{AW}: Alimentation dilution water flow [m3/h], f°_{FM}: Fermented must flow [m3/h], f°_{YC}: Treated Yeast cream flow [m3/h], f°_{W}: Wine flow [m3/h] and f°_{YCW}: Yeast cream dilution water flow [m3/h].

2.2 Analytical Methods

Analytical determinations of all subproducts were performed in an ISO 9001:2008 certified laboratory in compliance with established standards to ensure highest quality of the data obtained. Determination of Total Reducing Sugars (TRS) in raw materials (cane molasses and cane juice) and alimentation must was based on titration with Fehling solution and modified Eynon-Lane methods [15] [16]. Reducing substances (unfermentable) were measured in samples of molasses according to AOAC [17]. The Residual Sugar content at the end of the fermentation process was analyzed using the colorimetric method of dinitrosalicylic acid (DNS) calculating sugar concentration by extrapolation using standard curves [18]. Ethanol concentration of the fermented samples was measured with a Kjeldahl distillation apparatus (BÜCHI B-324) and Density Meter (Rudolph DDM2911)[15]. Titrable acidity was analyzed by titration with a sodium hydroxide solution and phenolphthalein as the indicator[15]. Glycerol was assayed using TG color Kit, Wiener lab (Enzymatic method for the determination of triglycerides in serum or plasma). A refractometer (Leica AR 600) was used for quantification of total diluted solids (ºBrix) [19] and a quick method was employed for estimating the percentage of yeast, by centrifugation (THERMO SCIENTIFIC, Sorvall Legend 1.6)[15].

Parameters were presented as the mean, standard deviation and coefficient of variation. Student parametric test (t-test) was used to compare the different methods. The software INFOSTAT [20] was used for all statistical analyses.

3 Sensitivity Study of the calculation Methods

The sensitivity study consisted in modifying each of the input parameters of the fermentation model with 5 or 10 % of the original value and then recalculating the fermentation efficiency using both the direct and indirect calculation methods described. The effect of the error in the input parameter (that is either 5 or 10 %) was calculated by comparing the original fermentation efficiency (0% error) with the recalculated value.

The effect of each parameter was classified according to their influence on the calculated fermentation efficiency where sensitivity levels were divided into 4 categories: "No influence": 0.4% or less change in the fermentation efficiency, “Low Influence ": change from 0.4 to 1%, "Intermediate Influence": change between 1 and 5% and "high-impact influence”: changes greater than 5%.

RESULTS

4 Mathematical Models

4.1 Fermentation Efficiency by Traditional Methodology (Direct Method)

The direct method, also known as the "traditional efficiency method", determines the efficiency (E_d) of a fermentation process by calculating the relationship between the ethanol concentration produced (P) and the fermentable sugar concentration entered (S) divided by the theoretical yield (0.6475 [L ethanol/kg sugar]). By combining equations 1, 2 and 3, a simplified formula was obtained as shown in Equation 10:

$$E_d = 154.44 * \frac{(f^{\circ}_W * p_W)}{\left(\left(f^{\circ}_M * \rho_M * (ts_M - us_M)\right) + \left(f^{\circ}_J * \rho_J * ts_J\right)\right)}$$

Equation 10

Where, f°_W: Wine flow [m3/h], p_W: Wine ethanol concentration [mL/100mL], f°_M: Molasses flow [m3/h], ρM: Molasses density [Kg/L], ts_M: Molasses total reducing sugars [g/100g], us_M: Molasses unfermentable sugars [g/100g], f°_J: Cane juice flow [m3/h], ρ_J: Cane juice density [Kg/L], ts_J: Cane juice total reducing sugars [g/100g].

The calculation of fermentation efficiency by the traditional method is a simple and useful methodology that evaluates how much ethanol is produced (ethanol output) compared to how much sugar is consumed.

4.2 Fermentation Efficiency by By-products Methodology (Indirect Method)

Another more accurate way to calculate the efficiency of a fermentation process is the Indirect Method. This method considers the production of by-products in the fermentation process, which can be directly measured, calculating the losses from the theoretical ethanol production in order to determine the overall efficiency of the fermentation process.

From the mass balances presented, a factor that considers all numerical constants of the formula was calculated. The final formula for calculation of industrial efficiency by the **indirect method adapted** in this work was as follows:

$$E_i = \frac{195.69}{(2 + 0.38 * K_X + 1.27 * K_G + 0.01 * K_A + 1.27 * K_R)}$$

Equation 11

Fermentation efficiency by measuring production of by-products (E_i) can be calculated using the equations shown above, where K_X, K_G and K_A are mass ratios between metabolites produced by yeasts and bacteria from available fermentable sugars (cellular biomass, glycerol and acids generated) and the product concentration of interest (ethanol generated during the alcoholic fermentation), where K_R is the relationship among residual reducing substances mass and ethanol mass output.

Where:

$$K_X = \frac{(f^{\circ}_{FM} * x_{FM}) - (f^{\circ}_{YC} * x_{YC})}{(f^{\circ}_{FM} * p_{FM}) - (f^{\circ}_{YC} * p_{YC})}$$

Equation 12

$$K_G = \frac{(f^{\circ}_{FM} * g_{FM}) - (f^{\circ}_{YC} * g_{YC})}{(f^{\circ}_{FM} * p_{FM}) - (f^{\circ}_{YC} * p_{YC})}$$

Equation 13

$$K_A = \frac{(f^{\circ}_{FM} * a_{FM}) - (f^{\circ}_{YC} * a_{YC}) - (f^{\circ}_{A} * a_{A})}{(f^{\circ}_{FM} * p_{FM}) - (f^{\circ}_{YC} * p_{YC})}$$

Equation 14

$$K_R = \frac{(f^{\circ}_{FM} * r_{FM})}{(f^{\circ}_{FM} * p_{FM}) - (f^{\circ}_{YC} * p_{YC})}$$

Equation 15

The symbols are: f°_{FM}: Fermented must flow [m3/h], p_{FM}: Fermented must ethanol concentration [mL/100mL], x_{FM}: Fermented must cells concentration [mL/100mL], g_{FM}: Fermented must glycerol concentration [g/100mL], a_{FM}: Fermented must acid concentration [mg/100mL], r_{FM}: Fermented must residual sugar concentration [g/100mL], f°_{YC}: Treated Yeast cream flow [m3/h], p_{YC}: Treated Yeast cream ethanol concentration [mL/100mL], x_{YC}: Treated Yeast cream cells concentration [mL/100mL], g_{YC}: Treated Yeast cream glycerol concentration [g/100mL], a_{YC}: Treated Yeast cream acid concentration [mg/100mL], f°_{A}: Alimentation must flow [m3/h] and a_A: Alimentation must acid concentration [mg/100mL].

5 Practical Application of the proposed Indirect Mathematical Model at Industrial Scale

To evaluate the applicability of the indirect model proposed for calculating the fermentation efficiency, a comparison among the obtained values from sampling of a continuous industrial scale ethanol production unit using Equation 11and those obtained using the traditional methodology (equation 10) was made. Calculations using the two formulas described in material and methods using values obtained from industrial scale sampling were applied and the average efficiency value and the coefficient of variation determined for both the direct and the indirect method. The efficiency values obtained applying the two calculation methods for each of the two sampling years of the ethanol production plant were compared using the Test T [20]. Both methods revealed significant differences in ethanol production efficiency between the two years of sampling (p value of 0.0005 for 2013 and 0.0392 for 2014).

In **Error! Reference source not found.**and **Error! Reference source not found.** all values used for calculating ethanol fermentation efficiency, by the direct (**Error! Reference source not found.**) and indirect (**Error! Reference source not found.**) methods for years 2013 and 2014 are shown. Values were obtained by laboratory determinations using the methods described in material and methods for each sample collected in the production plant during 2013 and 2014. In addition determinations of measured volume flows in the distillery as well as calculated fermentation efficiency are also presented.

The fermentation efficiency values calculated by the direct method for years 2013 and 2014 were 73.4% (CV = 1.8%) and 67.5 % (CV = 4.1%), respectively, whereas the values obtained using the indirect method were 78.6% (CV = 1.1%) for year 2013 and 71.3 % (CV = 1.2 %) for year 2014.

For the year 2013, diluted molasses was the only raw material used, whereas in 2014, both sugarcane juice and molasses were collected. As can be observed in table 1 the total sugar input was higher in 2014 as compared to 2013 but in spite of this, total alcohol produced was lower in 2014, 13,730.0 liters, compared to 14,064.2 liters in 2013 (**Error! Reference source not found.**). These results give a difference in sugar conversion yield into alcohol produced of 48% for 2013 and 44% for 2014 (data not shown).

In order to obtain data for the indirect method, major metabolites produced during the fermentation process (ethanol, glycerol and acids), the generated yeast biomass and the unfermented residual sugar (**Error! Reference source not found.**), were analyzed at the end of the fermentation process and the values obtained were used to calculate the efficiency of fermentation. By using this

method the ethanol produced by the distillery during the first year of sampling, 2013, was found to be higher than for the second year of sampling in 2014 (11,895.9 kg/h and 11,141.1 kg/h, respectively). The higher ethanol production obtained by the indirect method in the year 2013 coincides with the result of the direct method.

In accordance with the fermentation efficiency values obtained for the two years analyzed, the residual sugar mass in the 2014 sample was found to be higher (3,515.3 kg/h) in comparison with 2013 (3,028.6 kg/h). Regarding the yeast biomass generated during the two years studied, the 2014 sample (3,900.4 Kg/h) was two-fold the sample collected in 2013 (1,876.5 kg/h). Furthermore, the mass of glycerol was found to be 4-fold higher for the first year of sampling (617.5 kg/h) as compared to 2014 (157.0 kg/h), while the total acidity mass was 288.2 kg/h for the first sampling year and 737.5 kg/h for the 2014 (**Error! Reference source not found.**)

6 Sensitivity Study of the Mathematical Models

The sensitivity analysis was intended to evaluate the influence of a hypothetical error in the input value of all parameters involved in the fermentation efficiency calculations, either by the direct or by the indirect method.

For the direct method of calculation, except for non-fermentable sugars, all measurements of the products involved in the fermentation efficiency calculation showed an important effect on the final value of fermentation efficiency. This was clearly demonstrated by the sensitivity study performed, where either intermediate or high-impact influence was obtained for all parameters except unfermentable sugars, independent of the rate of error tested (5 or 10%)(**Error! Reference source not found.**).

In contrast, when testing the indirect calculation method, the sensitivity study indicated that no single input parameter showed a high-impact influence on the calculated fermentation efficiency. The most important effect was seen for yeast biomass and alcohol parameters in the fermented must, which showed an intermediate influence on the efficiency when using both 5% and 10% input errors., Residual sugars, yeast biomass and ethanol measured in the fermented must showed a similar influence but only when an input error value of 10% was used whereas a 5% input error only generated a low impact. Finally, the cream flow, the fermented must flow and the yeast concentration in the cream showed a low impact on the calculated efficiency when using the higher error value (**Error! Reference source not found.**).

DISCUSSION

Improvements of the industrial fermentation process in Brazil during the last 30 years has incremented the fermentation yield from 75-80%, at the beginning of the "ProAlcool" program, to allow yields as high as of 92–93%. This yield is referring to total ethanol production from sugars; however, as stated previously, yeast cells also produce glycerol, cellular biomass and organic acids during the alcoholic fermentation process. The formation of these by-products is the reason why industrial processes can only achieve a maximum of 92–93% of the theoretical ethanol production yield as the other 7–8% is directed towards cellular metabolism [21].

An important issue for the sugar-alcohol industry in Tucumán is the need to modernize equipments and improve efficiencies in the ethanol production, which currently operates with fermentation yields 10 to 15 points lower than those reported recently in Brazil. The low efficiency previously reported of fermentation in Tucumán was confirmed in this study for both calculation methods used. The fermentation efficiency calculated for 2013 was 73.4% using the direct method and 78.6% when applying the indirect method while for the year 2014 the efficiency were 67.5% and 71.3% using the direct and indirect method respectively. Possible explanations for the difference in fermentation yield between 2013 and 2014 is the significantly increased yeast cell biomass and relatively high lactic acid concentrations, in 2014 as compared to 2013, indicating more sugar consumption for cell formation and the presence of a contamination of lactobacilli and/or wild yeast cells; discussed later in this section.

It is interesting to notice that the ethanol fermentation efficiency calculated by the indirect method were, for both sampling years, significantly higher than those obtained by the direct method. As the indirect method analyzes a much larger number of parameters compared to the direct method, it is plausible to assume that this method will provide a more representative result.

As evidenced by the low fermentation efficiency values obtained from the ethanol production process in the distillery, it is of the highest importance to develop a robust method for calculating the fermentation efficiency, such as the indirect calculation method presented, in order to have an efficient tool to optimize the fermentation process. By employing the indirect method it is possible to determine the stoichiometry of metabolites of the industrial fermentation process, which enables to rapidly identify, correct and solve a punctual problem and to recover a higher ethanol production yield. After optimization of the fermentation process monitoring of yield could be employed by using the easier to handle direct method of calculation until another loss of efficiency takes place and the indirect method should again be used in order to identify the problem with higher accuracy.

An ethanol fermentation efficiency of 80 to 85% and a productivity ranging from 4 to 8 g/L.h has previously been reported for an industrial scale continuous fermentation process, based on free yeast cells growing in a fermentation medium containing 140 to 160 g/L of total sugars, a cell concentration of 10 to 12 g/L and a residence time of 5 to 8 hours [22]. In Brazil, where fermentation efficiency can reach up to 90%, the fermentation process normally includes very high yeast cell densities (10–15% wet weight basis/v) to ferment broths (cane juice and/or diluted molasses) containing 150–200 g/L of total sugar (mainly sucrose), producing high concentration of ethanol at 8–11% (v/v) with high productivity efficiency (each fermentation cycle lasts only 6–11 h) [11]. In contrast, we observed that the industrial-scale continuous fermentation system analyzed in this work, using must of molasses and/or cane juice with initial sugar concentrations within the range reported in Brazil [11] but with an initial yeast concentration of only 6.0% (v/v) (data not shown), as was the case in 2013, only produced a final ethanol concentration of 6.8 % (v/v). However, when a higher initial yeast biomass of 9.2% (data not shown) was used in 2014 the ethanol titer increased to over 8 %. This result is in agreement to the statement that the amount of yeast cells must be within certain limits in order to be able to reach higher ethanol concentrations (>8%) [23].

Nevertheless, although a higher yeast cell concentration in the fermentation tanks reduces the total processing time, concentrations higher than 15% has been found to decrease cell viability, increase acid consumption in the cream treatment and reduce the fermentation yield. In addition, under these conditions high numbers of dead cells are recirculated, releasing vitamins, amino acids and minerals to the fermentation must, which can serve as a substrate for contaminating microorganisms. An important drop in ethanol fermentation efficiency is also observed if the initial concentration of yeast cells in the fermentation tanks is low [23]. This undesirable effect is due to that a large part of the sugar is used for cell biomass production instead of ethanol. By studying the effect of different cell densities and statistical analysis it has been shown that the optimal level of yeast cells in the fermentation tanks is around 12% [23]. In our study we found no measurable increase in cell density between fermentor tanks 1 and 5 in the first year of study (2013), when a low cell density was used (6% v/v). However, despite the fact that the distillery was using higher concentrations of yeast in the fermentation tanks in the second year of this study , the production of cell biomass was found to slightly increase between tank 1 (9,25%) and 5 (10,25%). The change in this parameter could reflect the lower ethanol fermentation efficiency obtained

during 2014, which at least partly could be explained by the diversion of substrate utilization for cell division.

Regarding production of metabolites, glycerol is quantitatively the most important subproduct from yeast alcoholic fermentation, after ethanol and carbon dioxide, maintaining the redox potential of the cell. Glycerol is normally found in the range of ~1.2–1.5%, in yeast cells encountering abiotic stress factors such as high osmotic pressure and/or high temperatures among others [6]. Therefore, abolishment or a substantial reduction in glycerol production during the fermentation process may lead to a significant increase in ethanol yield [24]. The low values of glycerol found in this work as compared with previously reported concentrations [6], could indicate a low stress pressure in the fermentation tank. This low stress pressure could be explained by the low sugar and ethanol concentrations encountered for both years of sampling in the distillery, and because the metabolism of fermenting cells is not inhibited by substrate or product accumulation in a continuous fermentation process [25]. Moreover, the higher levels of glycerol found in the fermentation tanks during the first year of sampling can be justified by the use of molasses as a raw material, which causes a higher osmotic pressure as compared to cane juice [23]. Although we found a higher concentration of glycerol in 2013 than in 2014, it did not impact on the ethanol fermentation efficiency value calculated which, could be explained by the relatively low glycerol concentrations found in both years of sampling. The latter explication is further supported by the sensitivity study of the indirect method which showed that increasing glycerol concentrations did not influence the calculation of the efficiency value.

It has been reported that lactic and acetic acids are important factors affecting the yield of alcohol in fermentations, which in turn has a major impact on distillery economy [6]. Yeast produce different organic acids during fermentation, but concentrations are relatively low compared to those produced by lactobacilli and other contaminating bacteria. When lactobacilli are active, the production of lactic and acetic acids substantially increases and often the high acid content causes the arrest, or dramatically slows down, the fermentation metabolism. Depending on the nitrogen source used in the must, acid contents normally vary from 0.5 to 1.4 g/L but can rapidly rise to over 15 g/L under bacterial or wild yeast contamination. An acid content of 286.35 mg/100mL in 2013 and 522.10 mg/100mL for 2014 indicate a possible low to moderate bacterial and/or wild yeast infection in the fermentation system. Although a possible contamination was observed these low values did not negatively affect the ethanol fermentation efficiency as seen from the sensitivity studies.

Ethanol production is strongly negatively influenced by the unfermented sugar remaining as a consequence of an unfinished fermentation process, when the wine is delivered for distillation. We reported here concentrations of residual sugars of 1.25% (3028.6 kg/h) and 1.88% (3515.3 kg/h) in the fermented must for the 2013 and 2014 samples, respectively. The lower residual sugar found in the fermentation tanks in 2013 is in accordance with the higher ethanol production yield found that year, although lesser initial sugar concentrations were used.

In order to be able to evaluate the sensitivity of the two mathematical models, modified parameters were introduced on purpose in both equations and the impact of this error in the calculation of the ethanol fermentation efficiency was analyzed. In conclusion our results show that the indirect method is much less sensitive to an erroneous input of a specific parameter. This difference in sensitivity is understandable as the direct method calculates the fermentation efficiency considering only the input and output flow parameters, while in contrast, the indirect method also takes into account several possible metabolic deviation routes of the yeast cell by considering the production of metabolites as well as the remaining unfermented sugar.

CONCLUSIONS

This paper presents and compares two methods of ethanol fermentation efficiency calculation where the traditional calculation method or direct method was compared to an indirect method, analyzing the formation of different by-products of the fermentation process. The indirect method presented here is a modified version of an efficiency calculation method for continuous fermentation process previously described as "the method of losses".

The traditional calculation method (DM) is easier than the indirect method (IM) as it only requires carrying out a few determinations of input and output flows of the process. However, a minor error in anyone of the measured parameters will directly affect the calculated fermentation efficiency value. The indirect method of calculation requires a greater number of determinations, which makes it more complicated and time consuming; however it is much more robust since an error in any parameter will have a minor effect on the calculated fermentation efficiency value.

Based on the results obtained in this study, we recommend the use of the indirect calculation methodology in order to evaluate the real situation of the process and to reach an optimum fermentation yield for an industrial scale ethanol production. Once a high fermentation yield has been reached the

traditional method should be used to maintain control of the process. Upon detection of lower yields the indirect method should once again be employed as it permits a more accurate diagnosis of the causes of the change in yield reduction which is important to be able to correct the problem rapidly.

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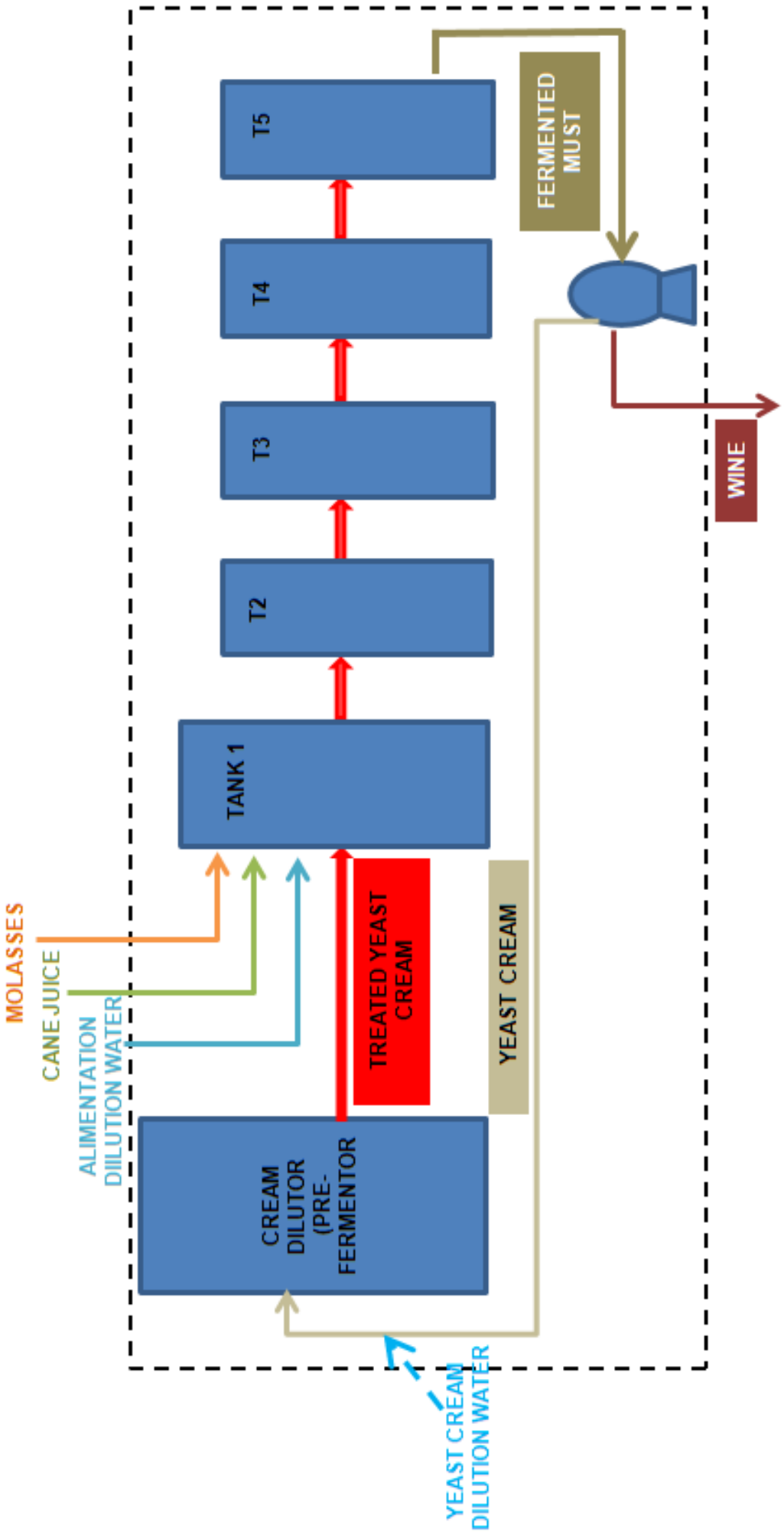
Figure 1


T5
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FERMENTED MUST
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MOLASSES
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YEAST CREAM
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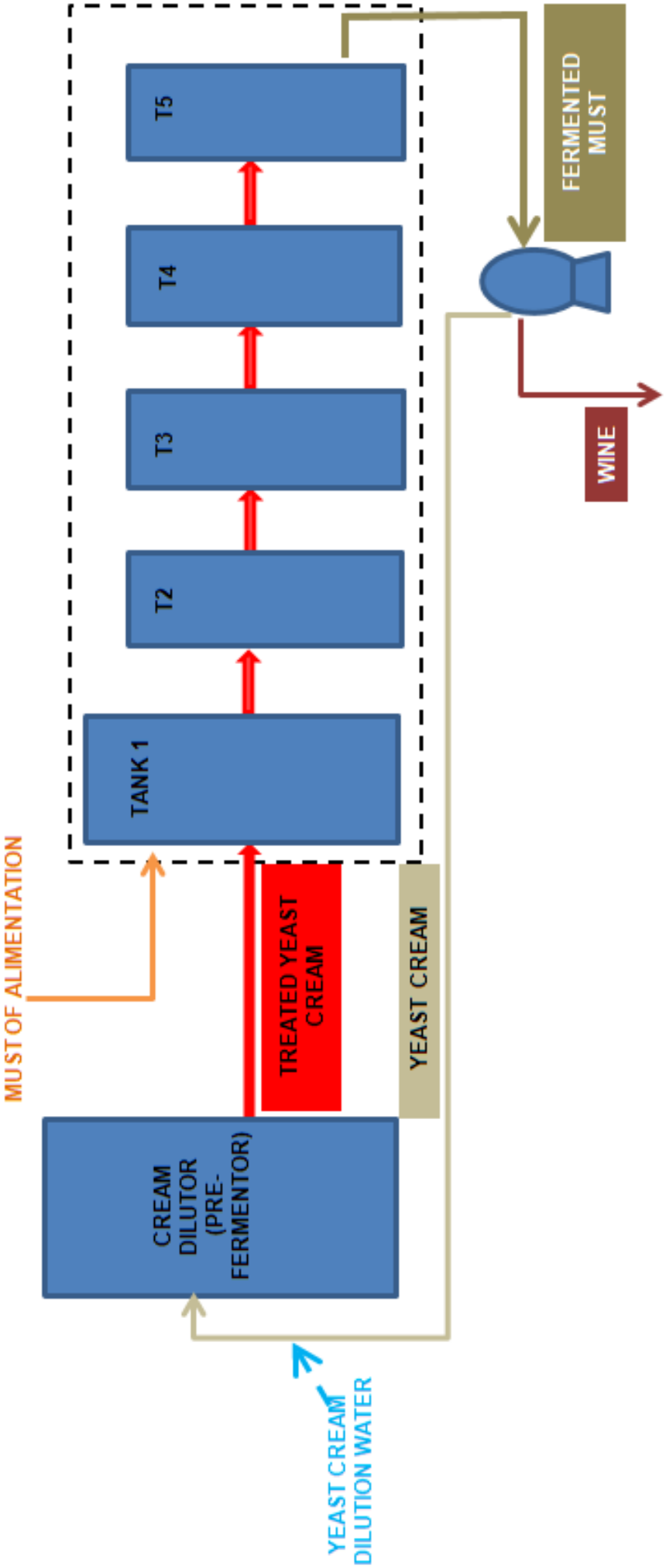
YEAST CREAM DILUTION WATER

Figure 2


T5
T4
T3
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TANK 1
FERMENTED MUST
WINE
MUST OF ALIMENTATION
TREATED YEAST CREAM
YEAST CREAM
CREAM DILUTOR (PRE-FERMENTOR)
YEAST CREAM DILUTION WATER

Table 1: Sample measurements and fermentation efficiency values obtained by the Direct Calculation Method

	10/31/2013		09/01/2014	
Determination on Samples	**Average**	**Coefficient of Variation**	**Average**	**Coefficient of Variation**
% Molasses Unfermentable Reducing Sugars (g/100g)	2.57	1.88	1.63	5.76
% Molasses Totals Reducing Sugars (g/100g)	65.68	1.36	66.3	0.88
Molasses Flow (m^3/h)	33.5	1.3	15.6	13.6
Cane Juice Density (g/ml)	N/D	N/D	1.06	0.01
% Cane Juice Totals Reducing Sugars (g/100g)	N/D	N/D	14.85	4.04
Cane Juice Flow (m^3/h)	0	N/D	110.6	2.9
Alimentation Dilution Water Flow (m^3/h)	153.9	0.4	18.3	0.3
Treated Yeast Cream Dilution Water Flow (m^3/h)	30.7	0	23.7	3.2
% Wine Ethanol Concentration (ºGL)	6.45	1.55	8.17	1.32
Wine Flow (m^3/h)	218.1	0.2	168.2	3.3
Ethanol Flow Generated (L/h)	14064.2	1.5	13730.0	2.4
Sugar Mass Flow Input (kg/h)	29593.8	1.0	31470.7	5.8
EFFICIENCY (%)	**73.4**	1.8	**67.5**	4.1

Table 2

Table 1: Sample measurements and the fermentation efficiency values obtained by the Indirect Calculation Method

	10/31/2013		09/01/2014	
Determination on Samples	**Average**	**Coefficient of Variation**	**Average**	**Coefficient of Variation**
Molasses Flow (m3/h)	33.5	1.3	15.6	13.6
Cane Juice Flow (m3/h)	0	N/D	110.6	3.0
Alimentation Dilution Water Flow (m3/h)	153.9	0.4	18.3	0.3
% Alimentation Must Acid Concentration (mg/100mL)	145.32	0.63	126.06	7.20
Alimentation Must Flow (m3/h)	187.4	0.2	144.5	3.3
% Treated Yeast Cream Cells Concentration (mL/100mL)	15.00	0	14.50	6.90
% Treated Yeast Cream Glycerol Concentration (g/100mL)	0.12	13.89	0.06	14.17
% Treated Yeast Cream Acid Concentration (mg/100mL)	244.30	5.91	134.19	18.08
% Treated Yeast Cream Ethanol Concentration (ºGL)	2.60	8.88	2.52	4.58
Treated Yeast Cream Flow (m3/h)	55.4	6.5	42.7	5.8
Yeast Cream Dilution Water Flow (m3/h)	30.7	0	23.7	3.1
% Fermented Must Cells Concentration (mL/100mL)	6.00	0	10.25	4.88
% Fermented Must Glycerol Concentration (g/100mL)	0.28	17.91	0.10	17.29
% Fermented Must Acid Concentration (mg/100mL)	286.35	6.31	522.09	3.32
% Fermented Must Ethanol Concentration (ºGL)	6.80	0	8.12	1.75
% Fermented Must Residuals Sugars Concentration (g/100g)	1.25	9.23	1.88	4.02
Fermented Must Flow (m3/h)	242.8	1.4	187.2	2.9
Ethanol Mass Flow Generated (kg/h)	11895.9	1.6	11141.1	2.9
Cell Mass Flow Generated (kg/h)	1876.5	5.5	3900. 4	10.8
Glycerol Mass Flow Generated (kg/h)	617.5	20.3	157.0	16.4
Acid Mass Flow Generated (kg/h)	288.2	18.3	737.5	5.9
Residuals Sugars Mass Flow (kg/h)	3028.6	10.4	3515.3	5.7
EFFICIENCY (%)	**78.6**	1.1	**71.3**	1.2

Table 1: Sensitivity study for the direct method

DIRECT CALCULATION METHOD	EFFICIENCY VALUES				ERROR RATES OBTAINED			
	5%		10%		5%		10%	
	Visit 1	Visit 2	Visit 1	Visit 2	Average	Standard Deviation	Average	Standard Deviation
% Molasses Unfermentable Sugars	73.55	67.42	73.70	67.45	0.13	0.10	0.26	0.21
% Molasses Totals Reducing Sugars	69.77	65.86	66.48	64.42	-3.60**	1.91	-6.91***	3.56
Molasses Flow	69.9	65.9	66.7	64.5	-3.48**	1.82	-6.69***	3.39
% Cane Juice Totals Reducing Sugars	N/D	65.57	N/D	63.85	-2.68**	N/D	-5.23***	N/D
Cane Juice Flow	N/D	65.6	N/D	63.9	-2.68**	N/D	-5.23***	N/D
Wine Flow	77.06	70.75	80.73	74.12	5.00**	0	10.00***	0
% Wine Ethanol Concentration	77.06	70.75	80.73	74.12	5.00**	0	10.00***	0
Ethanol Flow Produced	77.1	70.8	80.7	74.1	5.00**	0	10.00***	0
Sugar Mass Flow Entered	69.9	64.2	66.7	61.3	-4.76**	0	-9.10***	0

Note: The sensitivity levels were divided into 4 categories: "No influence" values less than 0.4 percentage errors (without *), "Influence Low": errors from 0.4 to 1% (*), "Influence Intermediate" errors between 1 and 5% (**) and "high-impact" greater than 5% errors (***).

Table 4

Table 1: Sensitivity study of the indirect method

INDIRECT CALCULATION METHOD	EFFICIENCY VALUES				ERROR RATES OBTAINED			
	5%		10%		5%		10%	
	Visit 1	Visit 2	Visit 1	Visit 2	Average	Standard Deviation	Average	Standard Deviation
% Alimentation Must Acid	78.68	71.29	78.71	71.31	0.04	0.01	0.07	0.02
% Treated Yeast Cream Acid	78.66	71.27	78.68	71.28	0.02	0.01	0.03	0.02
% Fermented Must Acid	78.55	71.15	78.46	71.04	-0.14	0.03	-0.28	0.06
% Treated Yeast Cream Glycerol	78.65	71.27	78.66	71.27	0.08	0.01	0.01	0.01
% Fermented Must Glycerol	78.55	71.25	78.46	71.22	-0.07	0.06	-0.14	0.12
% Treated Yeast Cream Cells	78.97	71.48	79.31	71.70	0.36	0.08	0.73*	0.17
% Fermented Must Cells	78.06	70.60	77.50	69.95	-0.83**	0.14	-1.65**	0.28
% Treated Yeast Cream Ethanol	78.57	71.19	78.49	71.12	-0.10	0.01	-0.20	0.01
% Fermented Must Ethanol	79.45	72.27	80.19	73.20	1.22**	0.27	2.34**	0.52
% Fermented Must Residuals Sugars	78.24	70.86	77.84	70.46	-0.54*	0.04	-1.07**	0.09
Alimentation Must Flow	78.7	71.3	78.7	71.3	0.04	0.01	0.07	0.02
Treated Yeast Cream Flow	78.9	71.4	79.2	71.6	0.29	0.11	0.58*	0.21
Fermented Must Flow	78.3	71.1	78.1	71.0	-0.31	0.11	-0.58*	0.21
Ethanol Mass Flow Generated	79.4	72.2	80.1	73.1	1.13**	0.26	2.17**	0.50
Cell Mass Flow Generated	73.6	70.8	78.1	70.4	-3.52*	4.08	-0.94**	0.44
Glycerol Mass Flow Generated	78.6	71.3	78.5	71.2	-0.06	0.05	-0.13	0.11
Acids Mass Flow Generated	78.6	71.2	78.6	71.1	-0.08	0.05	-0.17	0.10
Residuals Sugars Mass Flow	78.2	70.9	77.8	70.5	-0.54*	0.04	-1.07**	0.09

Note: The sensitivity levels were divided into 4 categories: "No influence" values less than 0.4 percentage errors (without *), "Influence Low": errors from 0.4 to 1% (*), "Influence Intermediate" errors between 1 and 5% (**) and "high-impact" greater than 5% errors (***).