# Nutrients' supplementation impacts on alcholoic fermentation of corn and sugarcane mixed wort

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## Abstract

# Background

Corn and sugarcane account for the world's most used feedstocks for ethanol production. Although, a single fermentation line integrating both of these raw materials still require further investigations. Some studies suggest that nutrient deficiency or excess influences the yeast metabolism to convert sugar into ethanol. In that regard, this work aims to explore the nutrients' supplementation impacts on alcoholic fermentation of corn and sugarcane mixed wort.

# Results

Corn hydrolysate and sugarcane syrup presented a Carbon/Nitrogen ratio above the recommended levels for ethanol production, 229.2 C:N ratio and 322.7 C:N ratio, respectively. The nutrients with the highest impact on alcoholic fermentation were determined using one-way ANOVA and Tukey statistical test. Thus, Nitrogen was the nutrient with the highest positive impact on yeast specific growth rate (49%), technological yield (35%) and productivity (32%). Organic acids ranged from 0.2–1.4 $g.L^{-1}$, glycerol was between 4–8 $g.L^{-1}$ and mannitol was under 0.05 $g.L^{-1}$. All were under the estimated ranges for alcoholic fermentation.

# Conclusions

Among the nutrients studied, Manganese, Potassium and Nitrogen were the nutrients which significantly impacted the fermentative parameters for ethanol production. Although both feedstocks were poor in Nitrogen for alcoholic fermentations, Nitrogen concentration supplied (2,970mg/L) was enough to promote the yeast consumption of all total reducing sugars presented in the mixed wort. As a result, Nitrogen was the only nutrient which increased both yeast specific growth rate and ethanol production. Nevertheless, further studies should investigate an optimal Nitrogen concentration in order to decrease the glycerol and drive the yeast metabolism, preferentially, for ethanol rather than biomass production.

## 1. Introduction

In order to achieve the 2030 Agenda for sustainable development, the Sustainable Development Goals (SDG) were established and pledged by world leaders [1]. These goals include actions such as improving energy efficiency, promoting sustainable industrialization and efficient use of natural resources [2].

In contribution to the 2030 Agenda, the federal program RenovaBio was created in Brazil [3]. As the program aims to promote biofuel expansion in Brazil's energy matrix, a growth in corn ethanol is expected for the next few years [4].

Currently, corn represents 64% of the feedstocks used for ethanol production in the world while sugarcane is responsible for 26% [5]. Despite the widespread use of corn for ethanol production it is still considered less sustainable than ethanol production from sugarcane [4]. According to critics, that is because corn ethanol industry is largely fueled by non-renewable energy [6].

Therefore, sugarcane integration in corn ethanol plants can add some benefits for the corn ethanol industry. One of them is increasing the corn ethanol plant's sustainability [7, 8]. It is possible, by shifting the fossil fuel burning for the burning of sugarcane bagasse [9]. Once the sugarcane juice is integrated in the corn fermentation process its bagasse can be reused to provide energy for corn ethanol plants [8].

Besides that, sugarcane addition in corn wort seems to increase ethanol yield by around 5% [8]. This rise happens because sugarcane juice contributes with nutrients and sugars for yeast growing [10]. Nutrients like Nitrogen, Phosphorus, Potassium, Magnesium, Calcium, Zinc, Manganese, Cobalt, Iron, Copper and Sodium play important roles in the yeast metabolism [10, 11].

The relationship between carbon and Nitrogen is also investigated as a parameter for efficient alcoholic fermentation [12]. Into the cell, Nitrogen is involved in the synthesis of biomolecules that allows yeast maintenance and reproduction [13]. Thus, Nitrogen deficiency in the medium can result in stuck or sluggish fermentation, as a consequence, it can decrease the ethanol yield [14].

Furthermore, these nutrients required for yeast fermentation may be found in the wort under or above the required levels [15]. However, no study about the impacts of supplementing corn and sugarcane mixed wort was found. To address this issue, the present study aims to assess the nutrients' supplementation impacts on alcoholic fermentation of corn and sugarcane mixed wort by monitoring parameters such as ethanol yield, residual sugars, organic acids and the yeast specific growth rate. Eventually, corn hydrolysate and sugarcane syrup physicochemical characterization are also targeted as support analyses for the investigations in this essay.

# 2. Materials And Methods

*2. 1 Feedstocks*

Corn hydrolysate and sugarcane syrup were supplied by the Laboratory of Sucroenergetic and Bioenergy Technology (LTSBio), at the Agroindustry, Food, and Nutrition Department (LAN) of Luiz de Queiroz Agriculture College - ESALQ/USP/Brazil. Corn hydrolysate was obtained by enzymatic hydrolysis using alpha-amylase and amyloglucosidase enzymes at the LTSbio's pilot plant. Differently, sugarcane syrup was produced by sugarcane juice treatment in sugar and alcohol plants in Piracicaba/Brazil. Both raw materials were stored at - 4 °C in order to avoid sugar degradation.

*2. 2 Feedstock physicochemical characterization*

Corn hydrolysate and sugarcane syrup were analyzed in regard to its carbohydrate content, pH, density and the following nutrients: carbon, Nitrogen, Calcium, Magnesium, Manganese, Sodium, Potassium,

Phosphorus, Zinc, Copper, Iron and Cobalt.

*2.2.1 Determination of carbohydrate content*

Carbohydrate content was analyzed by ion chromatography [16] using the column Metrosep carb 2 - 250/4.0 coupled to an amperometric detector. Before injection, ultrapure water was added to the samples in 10 mL volumetric flasks to achieve a 1:250 dilution. Subsequently, the samples were filtered in a filter of 0.22 µm and analyzed.

The parameters used as well as their values to quantify carbohydrate content by ion chromatography is shown in Table 1.

Table 1 - Parameters to measure carbohydrates content by ion chromatography

| Parameter | Value |
|---|---|
| Eluent | 200 mmol/L of Sodium hydroxide and 10 mmol/L of Sodium acetate |
| Flow rate | 0.7 mL/min |
| Sample volume | 10 Ml |
| Injection volume | 5 µL |
| Run-time for standard | 22 min |
| Run-time for sample | 40 min |
| Column temperature | 35 °C |
| Maximum pressure | 20 MPa |
| Calibration range | 1-800 $mg.L^{-1}$ |
| Standards | Carbohydrates kit (Sigma-Aldrich) |

*2.2.2 Determination of carbon and Nitrogen content*

Nitrogen and carbon were evaluated by TOC analyzer (TOC-L, Shimadzu). The Total Nitrogen – Total Nitrogen calibration range was between 0.5-1,000 $mg.L^{-1}$. On the other hand, total organic carbon - TOC and inorganic carbon - IC were estimated in a calibration range from 10 $mg.L^{-1}$ to 1,000 $mg.L^{-1}$. Potassium biphthalate was used as calibration standard for TOC measurements while the IC was evaluated using Sodium bicarbonate and Sodium carbonate standards. Sodium nitrate was the calibration standard for TN measurements.

The samples were diluted to 1:1000 in volumetric flasks with ultrapure water. Subsequently, they were filtered by a 0.22 µm filter and their pH were adjusted to 2-3 by adding a drop of Sodium chloride solution

37% m/v.

*2.2.3 Determination of other nutrients*

The other nutrients: Calcium, Magnesium, Manganese, Sodium, Potassium, Phosphorus, Zinc, Copper, Iron and Cobalt were measured by inductively coupled plasma optical emission spectrometry - (ICP OES).

Corn hydrolysate and sugarcane were digested by wet oxidation in order to carry out the analyses in ICP-OES. The methodology applied was adapted from the method note compendium of the MARS 6 microwave (CEM) [17]. Nitric acid and hydrogen peroxide were used in the acid digestion until the samples became colorless and particle free [17]. After that, the solution acquired was completed with water up to 50 mL. Lastly, it was filtered in a filter of 0.22 µm and analyzed.

The calibration curves ranged from 0.01 to 10 $mg.L^{-1}$ for the nutrients evaluated. Commercial standards from (Specsol) at 1,000 $mg.L^{-1}$ were diluted with ultrapure water in order to prepare the calibration curves for ICP-OES analyses.

Density and pH were determined using a pH-meter and a densimeter, respectively.

*2.3 Fermentation broth*

The first step to prepare the fermentation broths was to filter the corn hydrolysate in a 0.75 micrometer sieve to avoid solids in the wort. Subsequently, the corn hydrolysate and the sugarcane syrup were diluted to approximately 121 $g.L^{-1}$ of the total reducing sugar (TRS). At the end, the mixed wort was obtained blending 80% v/v corn hydrolysate and 20% v/v sugarcane syrup.

*2.3.1 Experimental design and preparation of nutrients solutions*

No research about the impacts of supplementing corn and sugarcane mixed wort was found. In this sense, the nutrients and nutrients concentrations were selected by benchmarking the studies with sugarcane wort carried out by the company Fermentec and Santos [18, 19].

The experimental design to evaluate the nutrients' impacts on the alcoholic fermentation is shown in Figure 2. Twelve conditions were evaluated, which include the Control assay with no added nutrient, and the ones containing the nutrients supplemented. In the experimental design, fermentations in microplate for ELISA were performed to evaluate the yeast growth while the fermentations in falcon tubes were accomplished to assess the fermentative parameters such as ethanol yield and productivity.

The concentration of each nutrient supplemented into the mixed wort, as well as its chemical form supplied is detailed in Table 2.

Table 2 - Conditions evaluated in the mixed wort supplementation tests [18,19]

| Nutrient supplied | Chemical form supplied in culture media | Nutrient concentration ($mg.L^{-1}$) |
|---|---|---|
| Calcium | Calcium Sulfate | 120 |
| Magnesium | Magnesium Sulfate | 135 |
| Manganese | Manganese (II) Sulfate | 21.50 |
| Sodium | Sodium Sulfate | 200 |
| Potassium | Potassium chloride | 750 |
| Phosphorus | Potassium phosphate | 311 |
| Zinc | Zinc Sulfate heptahydrate | 5.25 |
| Copper | Copper Sulfate | 7 |
| Iron | Iron Sulfate | 0.20 |
| Cobalt | Cobalt (II) sulfate heptahydrate | 10 |
| Nitrogen | ammonium sulfate | 2,970 |
| Mixed wort | Control condition (no added nutrient) | - |

*2.4 Yeast culture (inoculum)*

The *S. cerevisiae* strain Ethanol Red™ was kindly provided by the Bioprocess Engineering Laboratory - BELa from the University of São Paulo, Brazil. Furthermore, it was stocked at - 80°C into 1 mL cryogenic tubes containing 80% v/v yeast cells in YPD broth and 20% v/v glycerol.

To prepare the fermentation pre-inoculum, 1 mL from the Ethanol Red kept in the cryogenic tubes was added in 100 mL of sterilized YPD broth (20 $g.L^{-1}$ bacteriological peptone, 20 $g.L^{-1}$ glucose and 10 $g.L^{-1}$ yeast extract). The previous inoculum was kept overnight in 250 mL conical flasks cultivated at 30°C [20], shaking at 180 rpm in rotary shaker. Thereafter, the pre-inoculum was transferred to another conical flask containing sterilized YPD broth. Finally, it was kept overnight under the same conditions above in order to obtain the inoculum [11].

*2.5 Alcoholic fermentation*

Alcoholic fermentation was conducted in microplates for ELISA to evaluate Ethanol Red's growth kinetics and in falcon tubes to assess the metabolites production. Furthermore, the fermentations were carried out with mixed wort without sterilization procedures. The influence of microbial contaminants in the fermentation was assessed by measuring the alcoholic fermentation subproducts: mannitol, succinic acid, lactic acid, acetic acid and glycerol.

*2.5.1 Fermentation in microplate for ELISA*

Fermentations in the microplate for ELISA were conducted in solid plates with 96 round bottom wells. The wells were filled with 150 µL of mixed wort, 40 µL of nutrient solution and 10 µL of yeast inoculum (which meant an initial yeast concentration around 0.2 absorbance).

The Infinite 200 PRO was the multimode plate reader used to evaluate the Ethanol's Red growth kinetics by measuring its absorbance at 600 nm. About the conditions settled, the growth kinetics were evaluated at 30°C, every 20 minutes for 18.3 hours. The equipment orbital shaking amplitude and duration was defined in 1 mm and 27 seconds, respectively.

*2.5.2 Fermentation in falcon tubes*

During the fermentations carried out in falcon tubes, glucose, fructose, sucrose and maltose were the TRS monitored at the beginning and at the end of each fermentation. The metabolites selected to evaluate the fermentation quality were ethanol, glycerol, mannitol, acetate, lactate and succinate.

Before starting, falcon tubes were filled with 15 mL of mixed wort and 5 mL of nutrient solution. In addition, 1 mL of Ethanol Red inoculum was added, in order to start the fermentation with a yeast concentration of 0.2 absorbance.

Carbon dioxide loss was monitored by measuring the falcon tube's mass. Besides that, the fermentation was stopped when variations in the falcon tube's mass were less than 0.02 grams.

Finally, the falcon tubes were kept at 30 ºC [15], shaking at 180 rpm in a rotary shaker. Aliquots of 3 mL were taken from each falcon tube to evaluate the TRS and metabolites. The aliquots were taken at the beginning and at the end of the alcoholic fermentations.

*2.6 Determination of ethanol, glycerol, organic acids and mannitol*

The Glycerol and mannitol produced in each fermentation was measured by ion chromatography according to the method developed to evaluate carbohydrates content (Table 1).

The ethanol and organic acids (lactic, acetic and succinic) were assessed by high-performance liquid chromatography (HPLC) using the column Bio-Rad Aminex HPX-87H coupled to a refractive index (RI) detector.

Before injection, ultrapure water was added to the samples in 10 mL volumetric flasks to achieve a 1:10 dilution. Subsequently, the samples were filtered in a filter of 0.22 µm and analyzed. The parameters settled up to conduct the analyses by HPLC can be seen in Table 3.

Table 3 - Parameters to measure organic acids by HPLC

| Parameter | Value |
|---|---|
| Eluent | 5 mmol/L of sulfuric acid ($H_2SO_4$) |
| Flow rate | 0.6 mL/min |
| Sample volume | 1 mL |
| Injection volume | 10 µL |
| Run-time for sample and standard | 30 min |
| Column temperature | 60 °C |
| Calibration range | 1-20 $g.L^{-1}$ |
| Standards | commercially standards (Sigma-Aldrich) |

Calculations related to the alcoholic fermentations are described in Table 4 [21,22]. Between these fermentative parameters, two ethanol yield were determined. The technological yield is more similar to what is performed in the factories to measure fermentation efficiency. Whereas, stoichiometric yield considers the balance of ethanol and total reducing sugar presenting in the wort before and after fermentation. As the primary goal in this research is defining the nutrients which could improve ethanol production, both ethanol yield

Stoichiometric and technological yield are the main response variables for applying results at higher levels. These scaling up criteria help to assess the nutrients' impact on alcoholic fermentations even when the initial TRS provided differ by more than 5% between the conditions.

Table 4 – Fermentative parameters

| Variable | Method |
|---|---|
| Alcohol content% (v/ v) | |
| Technological yield | |
| Stoichiometric yield | |
| Ethanol productivity | |

Wherein:
C[*ethanol, f*] = net concentration of ethanol in the fermented wort
C[*TRS, i*] = concentration of TRS in the wort
*t* = fermentation time
*2.7 Statistical analyses*

The statistical analyses and graphs were accomplished using python, the software excel and Minitab. Except those mentioned, all experiments were performed in triplicate and the results were expressed as averages followed by its standard deviation. Analysis of variance (ANOVA) combined with Tukey's post hoc tests (statistical significance analysis with alpha value of 0.05) were performed to determine the nutrients' impact on alcoholic fermentation.

# 3. Results And Discussions

*3.1 Feedstock physicochemical characterization*

Before starting alcoholic fermentation tests, this study carried out a physicochemical characterization of both feedstocks employed. The first analysis conducted was the carbohydrate content determination by ion chromatography.

The main fermentable sugar concentrations found in corn hydrolysate and sugarcane syrup is shown in Table 5. Values for TRS were calculated including the glucose, fructose, sucrose and maltose amounts. Once sucrose and maltose absorb a water molecule to break down into two monosaccharides, its contribution for the TRS value was added as their amount divided per 0.95 [23, 24].

Table 5 - Feedstock carbohydrate content determined by ion chromatography

| Analyses | Corn hydrolysate ($g.L^{-1}$) | Sugarcane Syrup ($g.L^{-1}$) |
|---|---|---|
| Glucose | 369.09 ± 9.93 | 34.11 ± 1.84 |
| Fructose | 5.15 ± 0.59 | 46.16 ± 0.39 |
| Sucrose | - | 1,317.03 ± 8.50 |
| Maltose | 17.99 ± 0.72 | - |
| TRS | 393.18 ± 10.94 | 1,466.62 ± 10.28 |

During corn enzymatic hydrolysis, the starch present in the endosperm is mostly converted into glucose by enzymes like alpha-amylase and amyloglucosidase. Consequently, glucose was the sugar from corn hydrolysate that presented the largest proportion of TRS composition, 94.1%. Maltose and fructose were quantified in minor proportions, 4.6% and 1.3%, respectively.

Regarding the sugarcane carbohydrate content, it was noted that sucrose was the sugar with the higher contribution to TRS composition, 94.5%. As expected, no maltose was found in sugarcane syrup [25,26]. Lastly, fructose represented 3.2% and glucose 2.3% of TRS composition.

Sugarcane syrup exhibited 73.3% higher TRS concentration than corn hydrolysate. This growth occurred because the sugarcane juice used to make the syrup was previously treated by heating, and thus concentrating the juice.

Other implications from this previous treatment were also observed in the results from the total organic carbon analyzer. Since sugarcane syrup was concentrated before, its parameters evaluated in Table 6 returned higher values than the parameters evaluated for corn hydrolysate.

As sugars are included in the organic carbon composts and the feedstocks were rich in sugars (Table 6), the total organic carbon - TOC in the samples were thousand times higher than the inorganic carbon - IC.

The optimum C:N ratio for ethanol production by *S. cerevisiae* in tapioca starch is described as 35.2 C:N ratio [12]. Taking this to account, C:N ratio analyses indicate that corn hydrolysate and sugarcane syrup are rich in carbon, nevertheless, they are poor in Nitrogen for ethanol production.

Table 6 - Feedstock carbon and Nitrogen characterization by total organic carbon analyzer. These analyses were performed in duplicate and the results were expressed as averages followed by its standard deviation

| Analyses | Corn hydrolysate | Sugarcane Syrup |
|---|---|---|
| Total organic carbon -TOC (g. $L^{-1}$) | 151.86 ± 0.17 | 450.16 ± 12.34 |
| Inorganic carbon - IC (g. $L^{-1}$) | 0.15 ± 0.01 | 0.16 ± 0.02 |
| Total carbon - TC (g. $L^{-1}$) | 152.01 ± 45.7 | 450.32 ± 12.4 |
| Total Nitrogen - TN (g. $L^{-1}$) | 0.66 ± 0.07 | 1.39 ± 0.15 |
| Carbon/Nitrogen - (C/N ratio) | 230.09 ± 25.17 | 323.86 ± 25.73 |
| Total reducing sugar/Nitrogen - (TRS/Nitrogen) | 595.73 ± 45.93 | 1,055.12 ± 102.80 |

In addition to carbon and Nitrogen, other nutrients can improve yeast growth and fermentation. However, when the parameters exceed yeast requirements, they can play a role in inhibiting yeast growth or ethanol production [27].

Comparing the results acquired in Table 7 with the recommended levels for yeast fermentation proposed in the literature, it was noted that some nutrients were out of recommendation range. Regarding both feedstocks, Phosphorus, Magnesium and Iron exceeded the recommended range [18,19] while Manganese and Cobalt were under the recommended levels.

The nutrient content described in Table 7 refers to the total concentration of each element analyzed. In addition, a TRS/Nutrient ratio calculated for both feedstocks are shown in Table 7. Based on the TRS/Nutrient ratio, it is possible to estimate the concentration of nutrients present in corn and sugarcane wort. Besides that, some nutrients may be present in the feedstocks in not assimilable forms for the yeast metabolism [28]. Finally, due to aspects such as variety of soil, seed and climate, the composition of corn hydrolysate and sugarcane syrup can vary when compared to other references [28].

Table 7 - Feedstock physicochemical characterization by inductively coupled plasma optical emission spectrometry - (ICP OES)

| Nutrient analyzed | Sugarcane Syrup (mg. $L^{-1}$) | Sugarcane Syrup TRS/Nutrient | Corn hydrolysate (mg. $L^{-1}$) | Corn hydrolysate TRS/Nutrient |
|---|---|---|---|---|
| Potassium | 1,633.9 ± 65.1 | 0.9 ± 0.0 | 702.5 ± 22.6 | 0.6 ± 0.0 |
| Phosphorus | 485.7 ± 18.2 | 3.0 ± 0.1 | 564.3 ± 11.7 | 0.7 ± 0.0 |
| Magnesium | 316.8 ± 31.6 | 4.6 ± 0.5 | 144.3 ± 7.1 | 2.7 ± 0.1 |
| Sodium | 277.4 ± 33.9 | 5.3 ± 0.6 | 67.8 ± 7.9 | 5.8 ± 0.7 |
| Calcium | 146.1 ± 5.5 | 10 ± 0.4 | 30.5 ± 3.0 | 12.9 ± 1.2 |
| Zinc | 11.4 ± 1.5 | 128.9 ± 16.1 | 1.5 ± 0.0 | 262.1 ± 0.0 |
| Iron | 11.4 ± 1.5 | 128.9 ± 16.1 | 2.2 ± 0.8 | 181.5 ± 65.5 |
| Copper | 12.3 ± 1.5 | 119.7 ± 16.1 | 2.7 ± 0.8 | 147.4 ± 42.2 |
| Cobalt | 9.6 ± 1.5 | 152.4 ± 26.9 | 2.3 ± 0.3 | 168.5 ± 22.7 |
| Manganese | 5.3 ± 0.0 | 279.3 ± 0.0 | 2.3 ± 0.6 | 168.1 ± 37.7 |

The pH value and density measured for corn hydrolysate and sugarcane syrup is illustrated in Table 8. The pH feedstocks were similar and they were slightly above the optimal pH for *S. cerevisiae* fermentation, which is settled between 4.0 - 5.0 [13].

Table 8 - Others feedstock physicochemical analysis

| Analyses | Corn hydrolysate | Sugarcane Syrup |
|---|---|---|
| pH | 5.80 ± 0.04 | 5,70 ± 0.02 |
| Density (kg/$m^3$) | 1.10 ± 0.01 | 1,380 ± 0.02 |

*3.2 Fermentation in microplate for ELISA*

The alcoholic fermentations in microplates for ELISA were monitored for 18.3 hours. After this time, the yeast reached the stationary phase for all conditions evaluated. Despite the variation observed in the initial absorbance, Ethanol Red presented a final absorbance up to 0.43 for all conditions. Thus, by making a correlation between dry mass and absorbance, it was possible to find about 15 mg of Ethanol Red biomass production based on dry mass.

In Figure 3, a comparison is shown between the growth profile of Ethanol Red in mixed wort supplemented with eleven different conditions and without supplementation. It was noticed that Ethanol Red was able to grow in all conditions. Both feedstocks were diluted to achieve approximately 121 $g.L^{-1}$ TRS, as a consequence, the nutrients presented in the mixed wort were in lower concentrations than those found in the feedstocks (Table 7).

As bioethanol is a primary metabolite, its production is associated with yeast growth [39]. It means that ethanol production profile tends to be similar to Ethanol Red growth kinetics. The growth kinetics of Ethanol Red for all twelve conditions evaluated is shown in Figure 3.

The specific growth rates obtained for Ethanol Red in mixed wort supplemented with eleven nutrients and with no supplementation is illustrated in Figure 4. According to the results from the statistical tests, Nitrogen, Cobalt, Sodium and Phosphorus expressed an increase in the yeast specific growth rate. Between them, Nitrogen was the nutrient with the highest positive impact in the yeast growth rate, up to 49%. These outcomes suggest that the nutrients mentioned above, mainly Nitrogen, might contribute to increasing the alcoholic fermentations' yield.

When yeast reaches higher specific growth rates, the fermentation time is reduced and the ethanol productivity is increased [27]. Furthermore, it allows the yeast to dominate the process and inhibit the activity from microbial contaminants [29,30]. Consequently, parallel reactions carried out by microbial contaminants are performed at lower rates and more sugar is converted to ethanol [29,30]. All these points mentioned above represent advancements for the ethanol industrial process.

Unlike Nitrogen, Calcium decreased the yeast growth rate more than 22%. Despite Calcium improving yeast tolerance to ethanol stress, there are discussions about its required levels for yeast growth [27,9,19]. Calcium roles in the cell are related to actions on membrane function and structure. Thus, when it exceeds the yeast requirement, Calcium interferes with the amino acid and Magnesium uptake, and blocks cellular processes involving $Mg^{2+}$ [27].

For yeast growth, Calcium recommended level is around 180 $mg.L^{-1}$ [27]. Taking into account the amount of this nutrient found in the feedstocks' characterization (Table 7), it is noticed that Calcium was present in the sugarcane syrup in higher amounts than the recommended levels.

*3.3 Fermentation in falcon tubes*

The fermentation was stopped when the variations in falcon tubes' mass were less than 0.02 grams. In that order, the alcoholic fermentations in falcon tubes lasted for 52 hours.

As the yeast was supplied with approximately 121 $g.L^{-1}$ of TRS, it was expected to find a theoretical ethanol concentration up to 61.8 $g.L^{-1}$. However, during alcoholic fermentation, sugar can be deviated to produce other subproducts such as glycerol, organic acids or biomass rather than ethanol. The theoretical equation proposed by Gay-Lussac to describe sugar conversion from corn and sugarcane in ethanol and

carbon dioxide is shown in Figure 5 [31]. In addition, it also summarizes cell metabolism during alcoholic fermentation [21,22,32].

The condition that came closest to the theoretical concentration of ethanol was mixed wort supplemented with Nitrogen. In this condition, the fermentation started with 123 $g.L^{-1}$ ± 2 of TRS. At the end, 53 $g.L^{-1}$ ± 1 of ethanol were produced and not all sugar consumed was converted to ethanol. As expected, part of the 99% TRS consumed were probably transformed into biomass, glycerol, storage carbohydrates and fermentation byproducts as succinic acid [10]. Parallel reactions such as Maillard reactions and those carried out by microbial contaminants may also have been responsible for sugar consumption [10].

When Nitrogen is available in the medium in assimilable forms, it allows the biosynthesis of new molecules for yeast multiplication [33,34]. Therefore, the yeast catabolism is accelerated in order to produce more ATP for cell maintenance and growing. As a consequence, more hexoses are converted and more ethanol and carbon dioxide are excreted in the medium. Peptides, proteins, polyamides, nucleic acids and vitamins are some of the biomolecules produced with the Nitrogen uptake by the yeast [34].

Nitrogen, Magnesium and Zinc deficiencies or Calcium excess are reported as the main factors to stuck or sluggish fermentations [10]. Nevertheless, the results indicate that only Nitrogen was required to avoid stuck and sluggish fermentations. This nutrient was the only one in which residual TRS found was almost zero (1 $g.L^{-1}$). All other conditions remained with more than 32 $g.L^{-1}$ of residual TRS. The ethanol and glycerol production as well as the initial and residual TRS in the twelve conditions evaluated is described in Figure 6.

In regard to the other metabolites measured in this study, it was noticed that glycerol reached the average value of 4 $g.L^{-1}$ ± 1.2 for all conditions, except for the mixed wort supplemented with Nitrogen, which was 8 $g.L^{-1}$ ± 0.4. In alcoholic fermentation, approximately 10% of TRS provided is converted to glycerol [9,36]. Since the fermentation in falcon tubes started with an average of 121 $g.L^{-1}$ of TRS, the values found are under the expected range (12 $g.L^{-1}$). Glycerol is an indicator of the osmotic stress upon the yeast [28]. Furthermore, glycerol is associated with cell growth, once it works as an electron acceptor to maintain the NADH redox balance in anaerobiosis [10,37]. In that order, these were the main reasons why glycerol content in the mixed wort containing Nitrogen was higher than the values found in the other conditions.

Taking into account the statistical tests, Calcium was the only nutrient which decrease both yeast specific growth rate and the fermentative parameters. Iron, Zinc, Copper and Calcium had a negative impact on at least one of the fermentative parameters calculated.

Besides the negative effects of Calcium upon the yeast metabolism, already discussed previously, excess Copper and Iron can promote cell death [9,27]. Their impact on the yeast can vary according to the strains [27]. Iron is an enzyme catalytic center and it acts as a cofactor in the yeast respiratory activity and growth [19]. However, excess Iron induces cell death, and it can reduce enzymatic activities from enzymes such as pyruvate and succinate dehydrogenases [27]. Lastly, Zinc can be toxic when accumulated in excessive

amounts in the cell. The causes of Zinc toxicity are related to metabolic pathways suppressions, competition with metals for enzyme active sites and improper binds with intracellular ligands [35,27].

Nitrogen, Manganese and Potassium were the nutrients with fermentative parameters statistically higher than the Control sample. As Nitrogen and Manganese increased the technological yield and productivity, these results reinforce the evidence that both feedstocks were with their carbon/Nitrogen ratio (Table 6) above the recommended levels for ethanol production, 35.2 C/N ratio [12]. The mixed wort supplementation with Nitrogen was the condition with the highest increases in the yeast specific growth rate (49%), productivity (32%) and the technological yield (35%). The Nitrogen stoichiometric yield was 5% lower than the wort with no supplementation because it was the only condition which promoted the consumption of more than 99% of TRS.

Taking into account the fact that the Nitrogen concentration added (2.97 $g.L^{-1}$) was enough to consume almost all TRS supplied, further research with less Nitrogen concentrations should be carried out. In that order, a Nitrogen concentration to decrease the glycerol produced and drive the yeast metabolism preferentially for ethanol production rather than biomass production could be established.

Regarding the stoichiometric yield only Manganese and Potassium had a positive impact in this parameter. Comparing the values with the current declared in the ethanol industry (90-92%), it is noticed that Manganese and Potassium (95%) presented higher values [38]. Manganese is reported as a cofactor to enzymatic activities and may stimulate yeast growth and fermentation [27]. About Potassium, it is involved in many activities of yeast's anabolism and catabolism [40]. This nutrient helps the cell to cope with osmotic stress and improves its tolerance to ethanol. In addition, Potassium acts as a cofactor, and it is associated with the uptake of nutrients like phosphate [27,40]. The stoichiometric yield, the technological yield and the productivity are shown in Figure 7 for all conditions evaluated.

When ethanol reaches concentrations higher than 10% w/v, it can provoke a reduction in the yeast metabolic activity [41]. In addition, it induces toxic effects against the structure of the cell membrane [28]. As a consequence, ethanol inhibits the yeast growth and glucose conversion to the desirable product. Despite wort supplementation with Nitrogen having reached the highest ethanol content (7% ± 0.1), it was under the described levels to avoid ethanol stress on the yeast.

Another major factor in alcoholic fermentation are the organic acids detected in the wine. Although yeast can produce lactic acid [36], most of the lactic acid produced comes from the microbial contaminants' metabolism, mainly *Lactobacillus* [31,32]. The concentrations of lactic acid measured during the fermentations were about 0.3 $g.L^{-1}$ ± 0.1, except for Phosphorus (0.6 $g.L^{-1}$ ± 0.1). This indicates that microbial contaminants or yeast metabolism did not deviate large amounts of sugar from producing ethanol to producing lactic acid. Another hypothesis is that during the sugarcane juice treatment to obtain syrup, the microbial load may have been reduced by the heating.

Acetic acid is produced by ethanol oxidation and it causes toxic effects to yeast [36,34]. However, this organic acid can be produced by the own *S. cerevisiae* metabolism or by microbial contamination. The

acetic acid measured was under the levels to avoid toxic effects to yeast [10]. Mixed wort supplemented with Nitrogen was the condition which presented the highest acetic acid concentration, 0.8 $g.L^{-1}$ ± 0.1 while all the others returned values equal or less than 0.2 $g.L^{-1}$ ± 0.2 of acetic acid.

Succinic acid is usually excreted in the medium by the yeast to inhibit bacterial growth [36]. The succinic acid concentrations evaluated in the fermentations were up to 1.4 $g.L^{-1}$ ± 0.2 which were under the estimated ranges for alcoholic fermentation [34]. The organic acids measured in the fermented mixed wort is shown in Figure 8.

In addition to organic acids, mannitol is also used as an indicator of microbial contaminants in fermented wort [31]. As the mannitol concentration in the fermented wort was less than 0.05 $g.L^{-1}$, it suggests that Ethanol Red was the dominant microorganism acting in the fermentation.

# 4. Conclusions

The feedstock physicochemical characterization showed that corn hydrolysate and sugarcane syrup presented a carbon/Nitrogen ratio above the recommended levels for ethanol production. As a consequence, the Nitrogen was the nutrient with the higher impact on the alcoholic fermentation followed by Manganese and Potassium. The mixed wort supplemented with Nitrogen increased the yeast specific growth rate (49%), the technological yield (35%) and productivity (32%). Further research should be carried out in order to find out a Nitrogen concentration which could decrease the glycerol produced and drive the yeast metabolism preferentially for ethanol rather than biomass production.

Calcium was the only nutrient which decreased both yeast specific growth rate and the fermentative parameters. Iron, Zinc and Copper had a negative impact on at least one of the fermentative parameters calculated.

In regard to the other metabolites measured, it was noticed that Nitrogen supplementation returned the higher glycerol content due to the higher levels of ethanol produced and cell growth. At the end, mannitol and all byproducts measured: glycerol and the organic acids (acetic, lactate and succinic acid) were under the estimated ranges for alcoholic fermentation. As a result, we conclude that most of the TRS provided was driven by Ethanol Red for its growing and ethanol production rather than by microbial contaminants in parallel reactions.

# Abbreviations

YPD
yeast extract peptone dextrose
TRS
total reducing sugar
ATP
Adenosine triphosphate

C
carbon
C/N
Carbon/Nitrogen ratio
ICP OES
inductively coupled plasma optical emission spectrometry
ELISA
Enzyme-Linked Immunosorbent Assay
SDG
Sustainable Development Goals
TOC
Total organic carbon
IC
Inorganic carbon
TC
Total carbon
TN
Total Nitrogen
LTSBio
Laboratory of Sucroenergetic and Bioenergy Technology
LAN
Agroindustry, Food, and Nutrition Department
HPLC
high-performance liquid chromatography
RI
refractive index
BELa
Bioprocess Engineering Laboratory
ANOVA
Analysis of variance.

# Declarations

### Authors' contributions

NDS, MRA, CGA and MPGB designed the study. DCRE supervised the study carried out. NDS performed the experiments, analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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### Competing interests

The authors declare that they have no competing interests.

### Availability of data and materials

The datasets used and analyzed in the current study are available from the corresponding author upon reasonable request.

### Consent for publication

This article is published under license to Brazilian Company of Research and Industrial Innovation - EMBRAPII and Sinochem Brazil. Despite this, the authors declare that they have no conflict of interest with this publication.

### Ethics approval and consent to participate

Not applicable.

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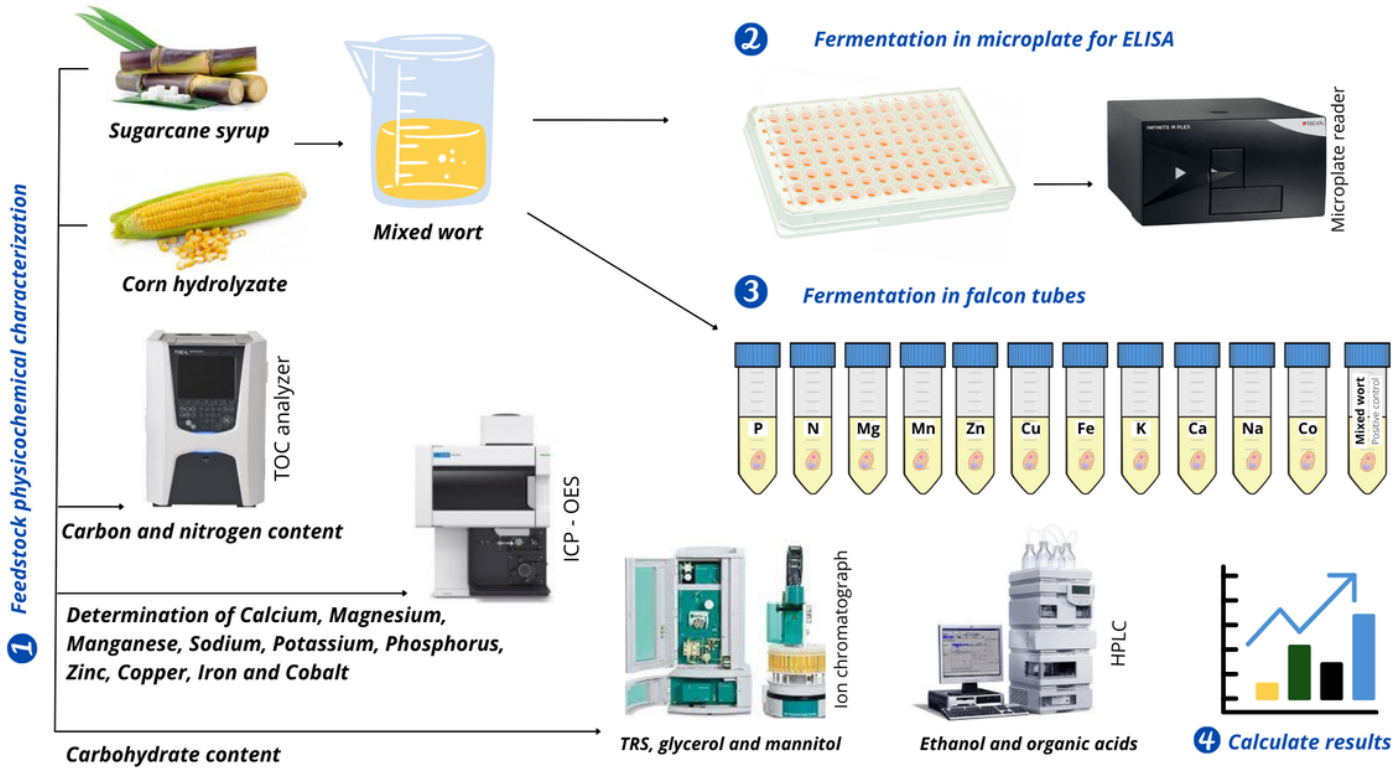
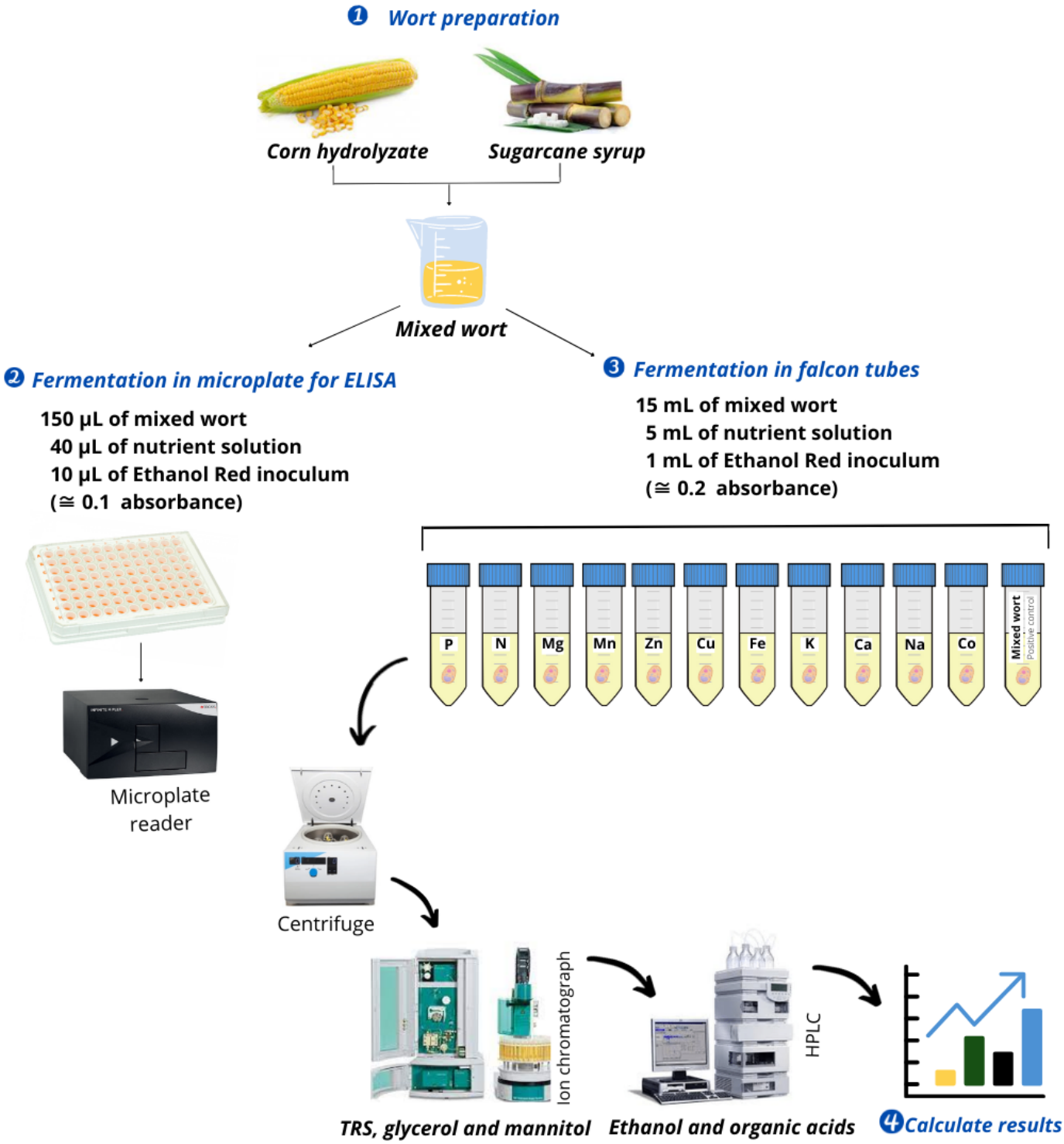
# Figures



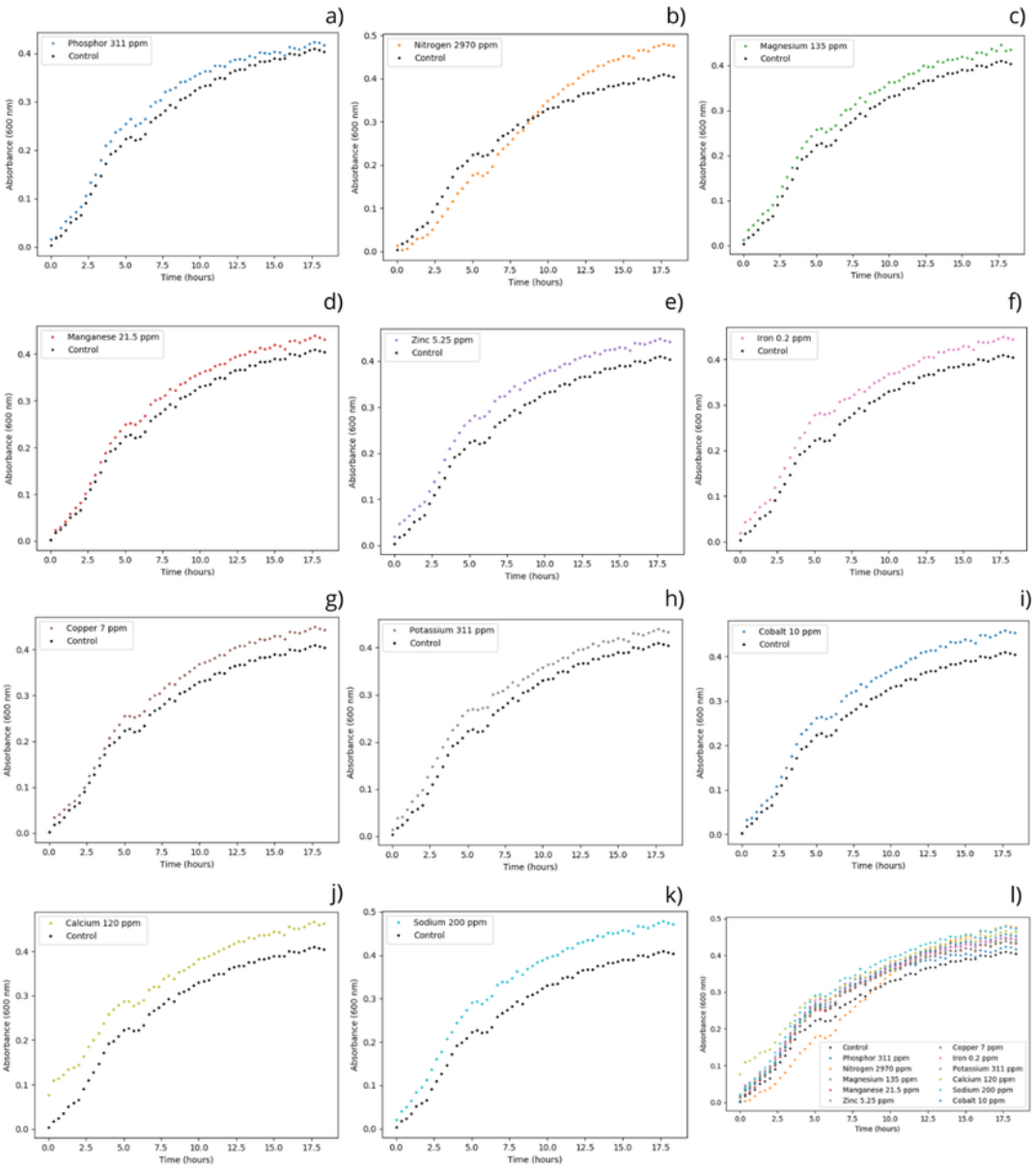

## Figure 1

Graphical abstract



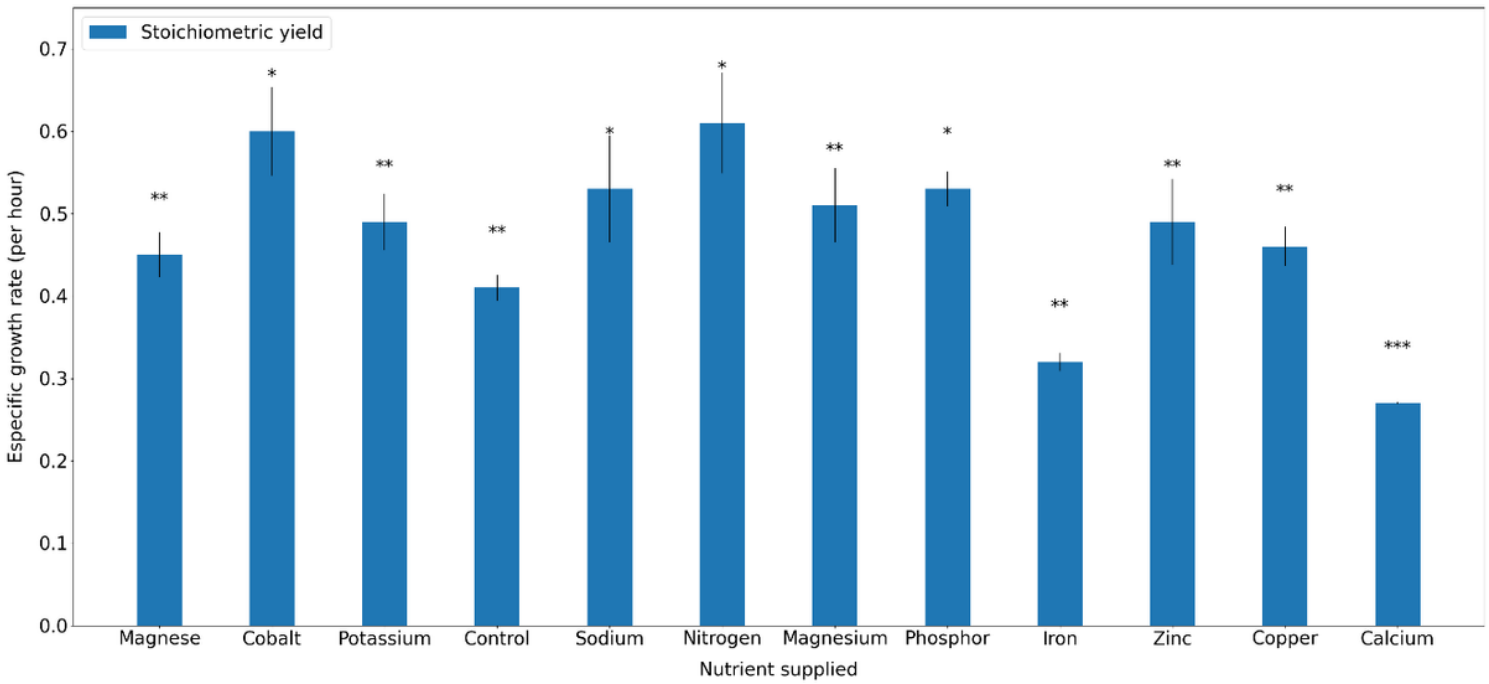

## Figure 2

Experimental design used to evaluate the nutrients' impacts on the alcoholic fermentation



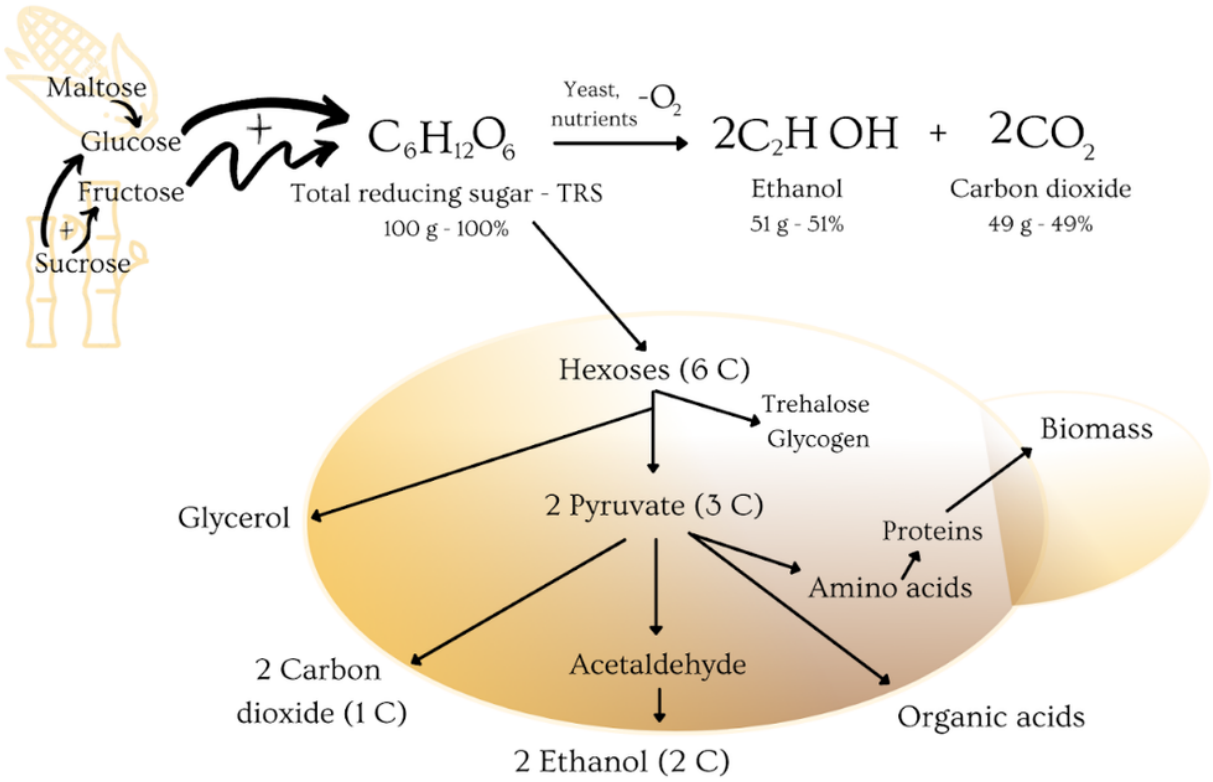

## Figure 3

Nutrients' supplementation impacts on the Ethanol Red growth kinetics. l) Ethanol Red growth kinetic was assessed in twelve conditions. The yeast growth profile in the mixed wort with no supplementation was compared to the yeast growth profile in the mixed wort supplemented with a) Phosphorus, b) Nitrogen, c) Magnesium, d) Manganese, e) Zinc, f) Iron g) Copper, h) Potassium, i) Cobalt, j) Calcium and k) Sodium.



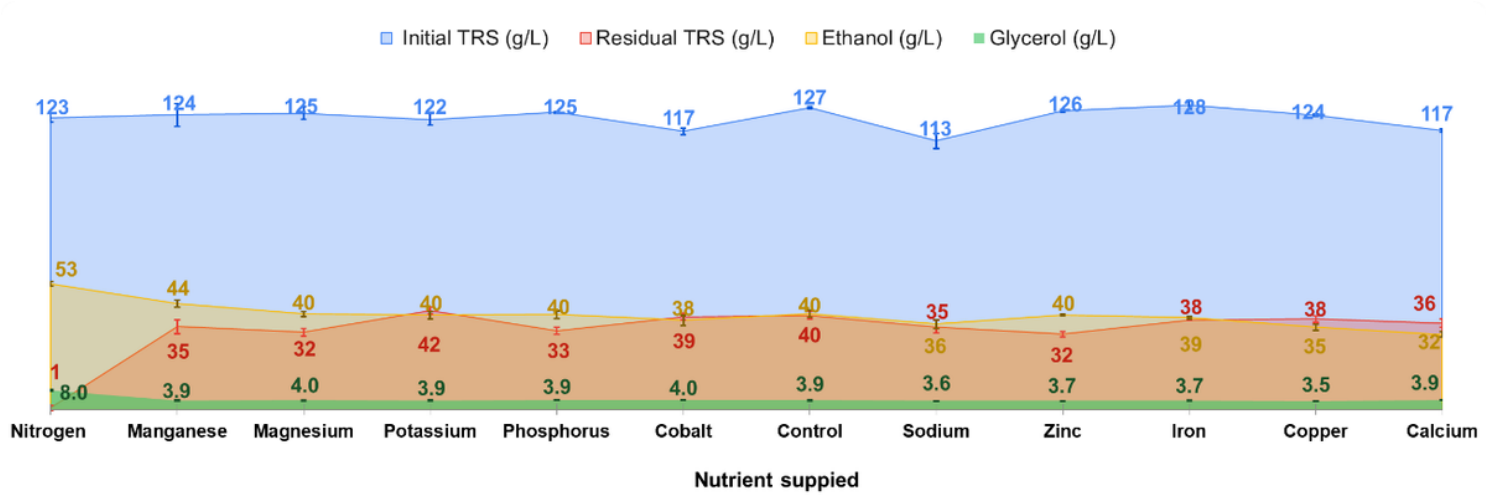

## Figure 4

Nutrients' supplementation impacts on the Ethanol Red specific growth rate. For specific growth rate values, asterisks indicate if averages are statistically higher (*), similar (**) or lower (***) than the Control sample.



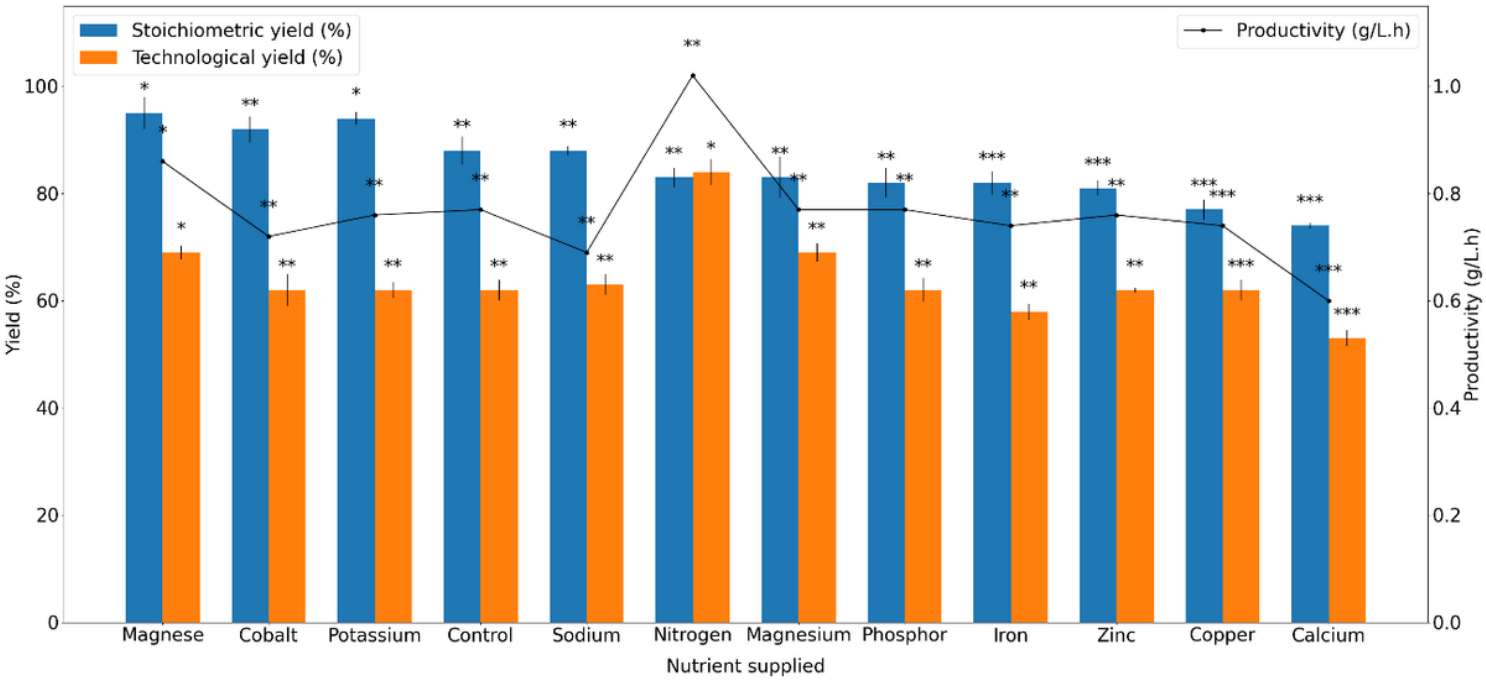

## Figure 5

Schematic representation of chemical equation carried out by the yeast in alcoholic fermentation as well as a summarized description of its metabolism during the process.



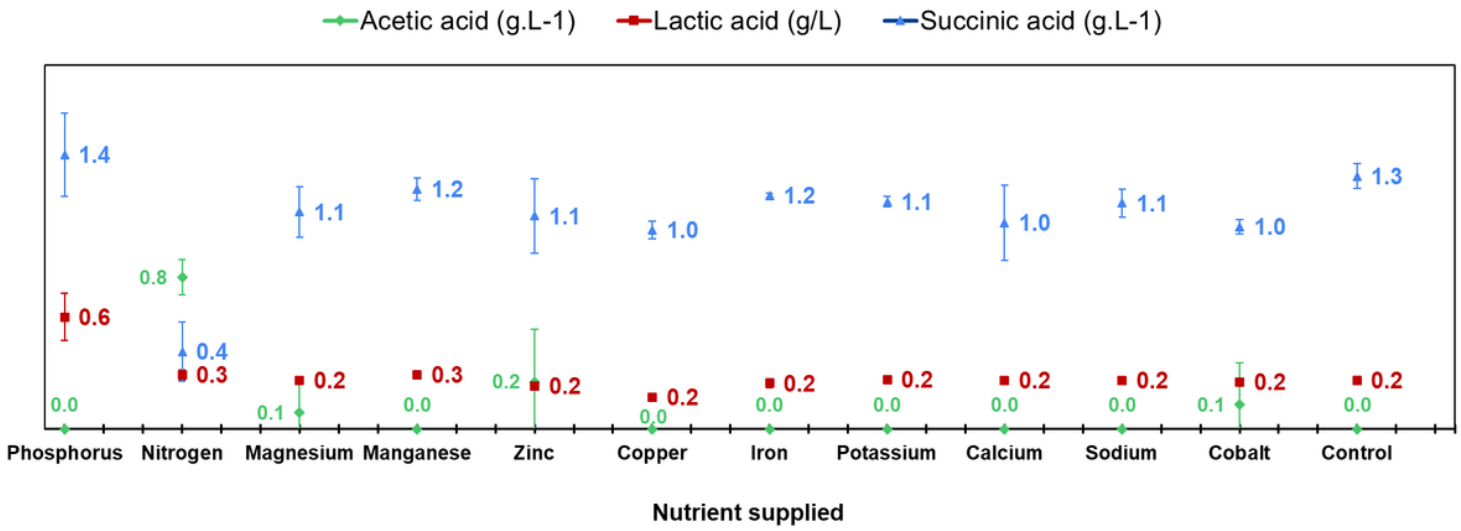

## Figure 6

Ethanol, glycerol and the initial and residual TRS in the wort mixed wort before and after alcoholic fermentation




## Figure 7

Nutrients supplementation's impacts on the alcoholic fermentation. For each response variable, productivity, stoichiometric and technological yield, asterisks indicate if averages are statistically higher (*), similar (**) or lower (***) than the Control sample.




## Figure 8

Organic acids concentrations in the fermented mixed wort

In addition to organic acids, mannitol is also used as an indicator of microbial contaminants in fermented wort [31]. As the mannitol concentration in the fermented wort was less than 0.05 $g.L^{-1}$, it suggests that Ethanol Red was the dominant microorganism acting in the fermentation.