

**UNIVERSITY OF SÃO PAULO**

**NATHÁLIA DIAS SILVA**

**ETHANOL PRODUCTION FROM CORN AND SUGARCANE MIXED WORT  
IRRADIATED BY ELECTRON BEAM**

São Paulo

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IRRADIATED BY ELECTRON BEAM**

**Corrected Version**

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Field of research: Chemical Engineering

Advisor: Prof. Dr. Marcela dos Passos Galluzzi Baltazar

Co-advisor: Prof. Dr. Denise Croce Romano Espinosa

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Assinatura do autor: Nathália Dias Silva

Assinatura do orientador: U. P. Sartazar

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For all women, black and first-generation college student.

For those who paved the way for me, and those who are counting on me to pave the  
way for them.

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*“If you are offered a seat on a rocket ship, don’t ask what seat! Just get on.”*

Sheryl Sandberg

## ABSTRACT

Concerns regarding climate change, and the RenovaBio program continue to drive the biofuel industry in Brazil, leading to an expansion of the corn ethanol sector in the coming years. Brazil, along with the United States (USA), holds the distinction of being the largest ethanol producers globally, with corn ethanol dominating in the USA and sugarcane ethanol in Brazil. However, despite their prominence, there is a lack of research focusing on the integration of corn and sugarcane in a single fermentation line. To address this issue, the present work aims to investigate the parameters involved in the ethanol production from corn and sugarcane mixed wort irradiated by electron beam, as bacterial contamination, and nutrient balance are among the key challenges faced in the industrial fermentation of these substrates. Corn hydrolysate and sugarcane syrup were characterized using techniques such as ionic chromatography, carbon organic analyser, and ICP OES. Additionally, alcoholic fermentations were carried out in microplate for ELISA, polypropylene 50mL conical tubes (Falcon tubes), and benchtop bioreactors. As the carbon/Nitrogen ratio in the corn hydrolysate (230.1) and sugarcane syrup (323.9) were above the required levels for ethanol production (35.2), Nitrogen was the only nutrient to avoid stuck fermentation, and returned the highest positive impact on yeast specific growth rate (49%), technological yield (35%) and productivity (32%). Regarding the wort decontamination tests, e-beam treatment at 15kGy achieved commercial sterilization, reducing 99.99% of the microbial load, while a higher dose of 20kGy was required to sterilize the mixed wort. The carbohydrate content and yeast viability remained unchanged after the electron beam treatment. Notably, the electron beam treatment at 15kGy and 20kGy resulted in accelerating biomass production, with yeast-specific growth rates increasing by 45.8% and 54.1%, respectively. Numerically, the application of e-beam at 15kGy, 20kGy, and Sodium Monensin 80% (Kamoran HJ at 3ppm) on mixed wort showed comparable results, with ethanol yield (both stoichiometric and technological) ranging from 90% to 92%. These values were higher than those observed in the control condition (86% to 88%). However, based on statistical analysis, the treatments did not show a significant increase in fermentation parameters in both tests carried out in conical tubes, and at bioreactor scale.

**Keywords:** alcoholic fermentation; electron beam; corn; sugarcane.

## RESUMO

As preocupações crescentes com as mudanças climáticas e a implementação do programa RenovaBio estão impulsionando a indústria de biocombustíveis no Brasil, especialmente o setor de etanol de milho. Embora o Brasil e os Estados Unidos sejam os principais produtores mundiais de etanol, utilizando cana-de-açúcar (BRA) e milho (USA), ainda são escassos os estudos que abordam os impactos da integração do milho e da cana-de-açúcar em uma única linha de fermentação. Desta forma, o presente trabalho visa avaliar parâmetros para aplicação de uma rota híbrida submetida a irradiação por feixe de elétrons na produção de etanol, uma vez que a contaminação bacteriana e o equilíbrio de nutrientes representam alguns dos principais desafios enfrentados na fermentação industrial destes substratos. O hidrolisado de milho e o xarope de cana-de-açúcar foram caracterizados usando técnicas como cromatografia iônica, analisador de TOC e ICP OES. Além disso, foram realizadas fermentações em microplaca para ELISA, tubos cônicos de polipropileno de 50mL (tubos Falcon) e biorreatores de bancada. Como a relação carbono/nitrogênio no hidrolisado de milho (230.1) e no xarope de cana-de-açúcar (323.9) estava acima dos níveis indicados para a produção de etanol (35.2), o nitrogênio foi o único nutriente capaz de evitar a fermentação incompleta e proporcionou o maior aumento na taxa específica de crescimento da levedura (49%), no rendimento tecnológico (35%) e na produtividade (32%). Em relação a descontaminação, o tratamento com e-beam a 15kGy alcançou a esterilização comercial, reduzindo 99,99% da carga microbiana. Entretanto, para esterilizar o mosto misto, foi necessária uma dose de 20kGy. O teor de carboidratos e a viabilidade celular da levedura não foram afetados pelo tratamento com feixe de elétrons. As doses de 15kGy e 20kGy aceleraram a produção de biomassa com o aumento da taxa específica de crescimento da levedura em 45,8% e 54,1%, respectivamente. Os resultados do rendimento de etanol foram semelhantes entre o tratamento com e-beam a 15kGy, 20kGy e monensina sódica 80% (Kamoran HJ a 3ppm) em mosto misto, variando entre valores de 90% a 92%, superando a condição controle (86% a 88%). No entanto, os tratamentos não apresentaram aumento significativo nos parâmetros de fermentação com base na análise estatística, tanto nos testes realizados em tubos cônicos quanto em escala de biorreator.

**Palavras-chave:** fermentação alcoólica; feixe de elétrons; milho; cana-de-açúcar.



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## LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
1G, 2G and 3G	First, second and third ethanol generation
Brazil	Brazil
BELa	Bioprocess Engineering Laboratory
C/N	Carbon/Nitrogen ratio
CFU	Colony forming unit
COP26	United Nations Conference on Climate Change 2021
CTR	Radiation Technology Center
DDGS	Distiller's Dried Grains with Soluble
DNA	Deoxyribonucleic Acid
e-beam	Electron beam
ELISA	Enzyme-Linked Immunosorbent Assay
ESALQ	"Luiz de Queiroz" College of Agriculture
EVs	Electric vehicles
FAO	Food and Agriculture Organization
HPLC	High-Performance Liquid Chromatography
IC	Inorganic carbon
ICP OES	Inductively coupled plasma optical emission spectrometry
IPEN	Nuclear and Energy Research Institute
LTSBio	Laboratory of Sucrenergetic and Bioenergy Technology
RDI	Radiation Dynamics Incorporation
RenovaBio	Acronym for National Biofuels Policy
SSF	Simultaneous saccharification and fermentation
TC	Total carbon
TN	Total Nitrogen
TOC	Total organic carbon
TRS	Total reducing sugars
UNICA	Brazilian Sugarcane Industry Association
USA	United State of America
USP	University of São Paulo
YPD	Yeast extract peptone dextrose

## 1 INTRODUCTION

### 1.1 Background

Concerns about climate changes have driven investments in the biofuels industry. Likewise, since 2011, electric vehicles (EVs) are seen as an alternative towards cleaner transportation<sup>1</sup>. During the COP26, in Glasgow, a declaration signed by automotive manufacturers and world leaders appears to accelerate the transition to EVs in the automotive market<sup>2,3</sup>.

Although the bioethanol industry may face substantial challenges, ethanol is still included in the global answer for climate changes and sustainability. In this regard, new technologies are being researched to replace grid electricity with ethanol to fuel electric vehicles. Looking to the future, aspects such as costs, infrastructure and technological issues will delay the large-scale implementation of EVs<sup>4</sup>.

Brazilian national policies are still driving investments in biofuels<sup>4</sup>. Holding a well-established bioenergy industry, Brazil launched in 2020 the RenovaBio policy as an environmental and economic strategy for the nation<sup>5,6</sup>. The RenovaBio targets contribute to the 2030 Agenda and promote a diversification in the country's energy matrix. In this sense, corn ethanol sector is expected to grow in the coming years<sup>7</sup>.

Ethanol from corn (USA) and ethanol from sugarcane (Brazil) rank these countries as the world's largest ethanol producers<sup>8</sup>. Nonetheless, there is a lack of studies on the impacts of integrating corn and sugarcane in a single fermentation line for ethanol production.

This hybrid process benefits are related to an increase in ethanol yields of up to 5% and improvements in the corn ethanol sustainability<sup>9</sup>. The energy acquired with the sugarcane bagasse burning can replace the fossil fuels burning in corn ethanol plants<sup>6</sup>. Furthermore, the nutrients presented in the sugarcane juice contribute to the yeast fermentation<sup>9</sup>.

Nutrients such as Potassium and Magnesium and the carbon/Nitrogen ratio play important roles in cell metabolism. It is noteworthy that some nutrients' absence or excess have been mentioned as the reasons for sludge and stuck fermentations<sup>10</sup>. As they can be found in different proportions in worts, investigations about the nutrients' impacts on alcoholic fermentation of mixed wort must be deepened.



When the sugarcane juice is added to corn wort, it brings not only nutrients, but also its microbial community. Nevertheless, the *bacillus* and *lactobacillus* bacteria are reported as the main microbial contaminants identified in both corn and sugarcane worts<sup>11,12</sup>.

The industrial fermentation process has several inputs for microbial contamination. Usually, when the contaminants reach concentrations higher than  $10^7\text{CFU.mL}^{-1}$ , they can decrease ethanol yield<sup>13,14,15</sup>. In that order, antibiotics such as penicillin, monensin, virginiamycin, tetracycline and streptomycin are implemented in the process (fermentation and milling steps) to maintain contaminants in lower concentrations<sup>16,17,18</sup>.

Antibiotics and other decontamination alternatives such as the chemical agents increase the bioethanol producing costs<sup>15,16</sup>. In addition, bacteria also develop resistance to antibiotics<sup>16</sup>. Thus, the energy surplus generated by burning sugarcane bagasse can be used to implement decontamination methods like the electron beam irradiation<sup>15</sup>. Electron beam is an ionizing radiation method produced in electron accelerators<sup>19</sup>. It can sterilize surfaces and wort by interactions with the DNA and other cell components from the microorganism's community present in the medium<sup>20,21</sup>.

e-beam irradiation has been used in numerous research and in automotive, aerospace, healthcare, food and environmental industries<sup>22</sup>. In USA, South Korea and Brazil, industrial plants using this technology showed the effectiveness of e-beam treatment<sup>23</sup>. The commercial e-beam facility in South Korea demonstrated a capacity to treat  $10,000\text{m}^3.\text{d}^{-1}$  of textile dyeing wastewater<sup>24</sup>. Moreover, a Brazilian plant showed to be cost competitive in treating  $3\text{m}^3.\text{h}^{-1}$  of wastewater and drinking water<sup>23</sup>. Lastly, the construction of an electron beam truck by the Energy and Nuclear research - IPEN will expand the electron beam industrial applications and research in Brazil<sup>25</sup>.

Therefore, studying about the effects of nutrients, wort decontamination and the process scale-up are important topics to create a single fermentation line to produce ethanol from corn and sugarcane mixed wort.

## **1.2 Structure of the master dissertation**

This dissertation was subdivided into four main topics: feedstock physicochemical characterization, assessment of nutrients' supplementation on alcoholic fermentation, effects of electron beam and antibiotic on wort

decontamination, and impacts of scaling up mixed wort fermentation. Notably, all results presented in this work were obtained from batch fermentations without yeast recycling and were conducted using a non-simultaneous saccharification and fermentation process (SSF). It is also imperative to highlight that Ethanol Red™ was the *Saccharomyces cerevisiae* strain employed for all fermentations. By highlighting these important aspects, the study's findings can be accurately interpreted and utilized to inform future research and practical applications. The mentioned topics are described below:

**i. Topic I:** Corn hydrolysate and sugarcane syrup were characterized regarding their physicochemical properties. In this sense, the carbohydrate content was determined by ion chromatography for both feedstocks. While carbon and Nitrogen were evaluated by total organic carbon analyzer- TOC, the other nutrients (Potassium, Phosphorus, Magnesium, Sodium, Calcium, Zinc, Iron, Copper, Cobalt, and Manganese) were measured by ICP OES. Lastly, the pH and density were also determined to set up the e-beam irradiation's parameters. In the end, the results for both feedstocks were compared.

**ii. Topic II:** Nutrients' supplementation impacts on alcoholic fermentation were studied in microplates for ELISA and in polypropylene 50 mL conical tubes (Falcon tubes). Fermentations in the microplate for ELISA were performed to evaluate the yeast growth profile and its specific growth rate. On the other hand, fermentations in conical tubes were carried out to study the nutrients impacts on the fermentative parameters such as ethanol yield, alcohol content and productivity. The fermentations in the mixed wort with no supplementation were compared to fermentations in the mixed wort supplemented with Phosphorus, Nitrogen, Magnesium, Manganese, Zinc, Iron, Copper, Potassium, Cobalt, Calcium, and Sodium.

**iii. Topic III:** Ethanol production was studied using mixed wort submitted to two decontamination treatments: electron beam irradiation at 10kGy, 15kGy and 20kGy doses and by the antibiotic Sodium monensin 80% (Kamoran HJ at 3ppm). As performed in the Topic II, alcoholic fermentations in microplate for ELISA and in conical tubes were carried out to evaluate the yeast growth and fermentative parameters. In addition, microbiological analyses and carbohydrate stability were conducted to assess the decontamination methods efficiency. All results were compared with the data achieved for the untreated mixed wort.

**iv. Topic IV:** Fermentations in bioreactors were conducted to evaluate the scale-up effects on the fermentative parameters such as ethanol yield and productive. Firstly, three conditions were tested, the mixed wort without treatment (control), treated by electron beam at 20kGy doses and with Kamoran HJ at 3ppm. Finally, fermentation was carried out in a bioreactor, integrating nutrient supplementation (Nitrogen, Potassium, and Phosphorus) and electron beam treatment at 20kGy, since these two conditions increased the fermentation parameters.

An illustrative scheme of this master dissertation is shown in Figure 1.

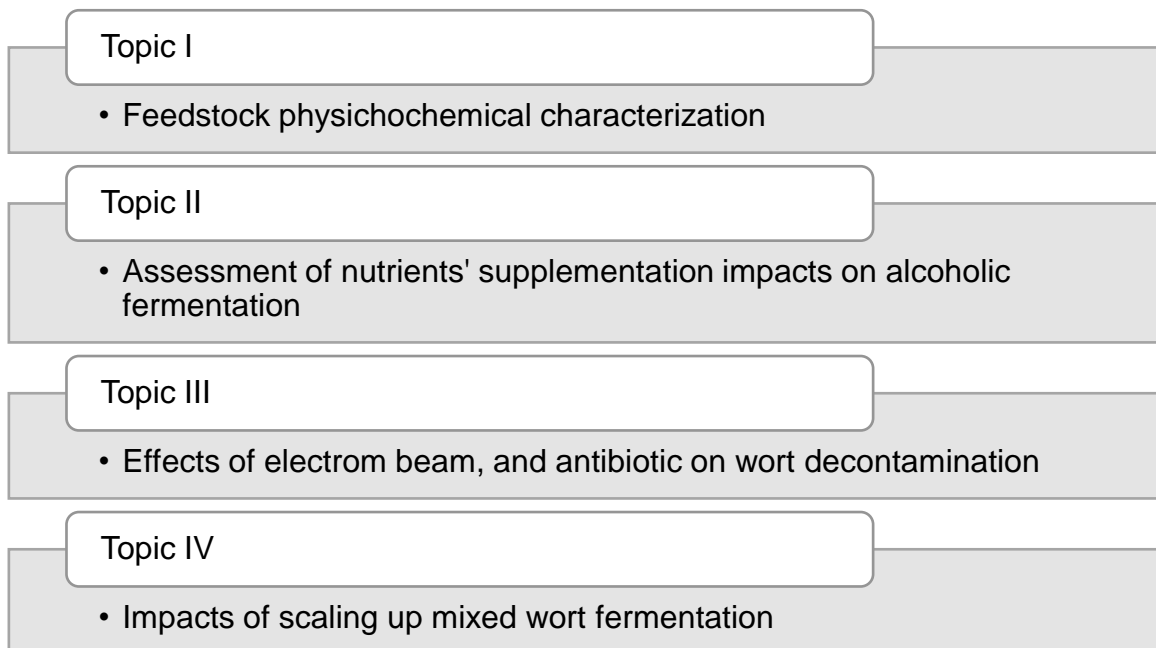


Figure 1 - Structure of the master dissertation

## 2 LITERATURE REVIEW

### 2.1 Ethanol generations

Advances in biotechnology have provided a broader range of renewable feedstocks for ethanol production. Thus, bioethanol is commonly classified as first (1G), second (2G), third (3G) and fourth (4G) generation according to the biomass and technology applied in the process.

Bioethanol production on a large scale primarily relies on first-generation methods, which involve utilizing food crops like sugarcane, corn, wheat, and other starch or sugar-based biomass<sup>26</sup>. Furthermore, countries in tropical and subtropical zones usually are sugar-based ethanol-producing, notably Brazil. Starch-based ethanol production is in greater numbers found in the United States, China, Canada, France, Germany, and Sweden, respectively<sup>27</sup>.

Looking at 2G ethanol, it is based on lignocellulosic biomass like straw and agricultural wastes. The second-generation is also an alternative for 1G once it does not rely on feedstocks from food crops<sup>28</sup>. Although its feedstocks are available in many countries, the 2G ethanol large-scale process is still more expensive than 1G<sup>27</sup>.

Third-generation ethanol emerged from studies on algae applications in biotechnology. Therefore, the fourth generation was created to refer to advances in microalgae genetic modification for ethanol production<sup>29</sup>. According to Suali and Sarbatly<sup>30</sup> dry weight algae contain up to 70% of lipid. Moreover, the cultivation' growth rate and the low cost are some aspects that drive research into algal biomass.

Industrial costs to produce ethanol varies according to the feedstock. In general, the feedstock represents about 40-75% of the total costs to produce ethanol<sup>31</sup>. The bioethanol production costs are still higher than gasoline<sup>32</sup>, except in Brazil that have the lowest cost of ethanol production worldwide. Its environmental benefits such as the reduction of greenhouse gas emissions are one of the reasons that justify the investment.

The industrial costs associated with ethanol production are contingent upon the type of feedstock utilized. Typically, feedstock expenses comprise a significant portion, ranging from 40-75%, of the total expenses incurred during the ethanol production process<sup>31</sup>. Although the production costs of bioethanol are typically higher than those of gasoline, Brazil is an exception, with the lowest ethanol production costs worldwide.

The cost of anhydrous ethanol in Brazil ranges from US\$ 0.22 to 0.33 per liter, while the cost of gasoline (100%) derived purely from oil and refining in Brazil is approximately US\$ 0.60 per liter (exclusive of taxes and distribution costs)<sup>33</sup>.

One of the primary driving forces behind investment in bioethanol production is its environmental benefits, particularly in reducing greenhouse gas emissions. These advantages have captured the attention of both academia and industry professionals, as the global community seeks sustainable alternatives to conventional fossil fuels. Extensive research in this field has explored various feedstocks and production methods to enhance the efficiency and cost-effectiveness of ethanol production. Presently, the global feedstocks for ethanol production are composed of 64% corn, 26% sugarcane, 3% molasses, 3% wheat, and the remaining portion comprises other raw materials such as grains, cassava, and sugar beets.

According to the Food and Agriculture Organization, sugarcane and corn will remain as the main feedstocks for bioethanol production in the next years<sup>8</sup>. In addition, it is estimated that in 2029, 25% to 14% of the world's sugarcane and corn production will go to the bioethanol industry<sup>8</sup>. The production share and the main feedstocks used by countries with the largest ethanol production in the world are exposed in Table 1.

Table 1 - Ethanol production share and major feedstocks, adapted from ref. (8).

<b>Country</b>	<b>Ethanol production share</b>	<b>Major feedstocks</b>
United States	48.2%	Corn
Brazil	26.2%	Sugarcane/corn
China	8.1%	Corn/cassava
European Union	4.9%	Sugar beet/wheat/corn
India	2.1%	Molasses
Canada	1.4%	Corn/wheat
Thailand	1.4%	Molasses/cassava
Argentina	0.9%	Molasses/corn
Colombia	0.4%	Sugarcane
Paraguay	0.4%	Sugarcane
Indonesia	0.2%	Molasses

## 2.2 Feedstocks

Ethanol production requires carbon-rich materials as feedstock in its processes. Thereby, different raw materials can be applied to acquire bioethanol, mainly those that provide readily fermentable sugar for the yeast metabolism<sup>34</sup>.

Raw materials containing polysaccharides such as starch and lignocellulose are also applied to produce bioethanol, however, they need to be pre-treated and hydrolysed to release the fermentable sugar<sup>35</sup>. In that order, the raw material must be converted into reducing sugars to become assimilable to the yeast.

Reducing sugars are defined as a category of carbohydrates that can act as reducing agents due to their free aldehyde (-CHO) or ketone (-CO-) groups. Their functional groups allow them to donate electrons to other species by redox reactions<sup>36</sup>.

All monosaccharides are considered reducing sugars, e.g., glucose, fructose, glyceraldehyde, and lactose. Some disaccharides, oligosaccharides and polysaccharides carbohydrates are also included in this category<sup>37</sup>.

Both maltose and sucrose are disaccharides. While maltose is included in the reducing sugar category, sucrose is classified as non-reducing sugar. Non-reducing sugars such as sucrose has the anomeric carbon involved in glycosidic bonds, which retain its cyclic structure and block its open chain form<sup>36</sup>.

Unlike sucrose, the anomeric carbon from maltose structure is not involved in glycosidic bonds, thus, it is free to form an open-chain structure. It is worth remembering that monosaccharides that contain ketones must be first tautomerized in aldoses to work as a reducing sugar<sup>37</sup>.

The chemical structures from reducing sugars such as glucose (monosaccharide), fructose (monosaccharide), and maltose (disaccharide) and non-reducing sugars as sucrose (disaccharide) are evidenced in Figure 2.

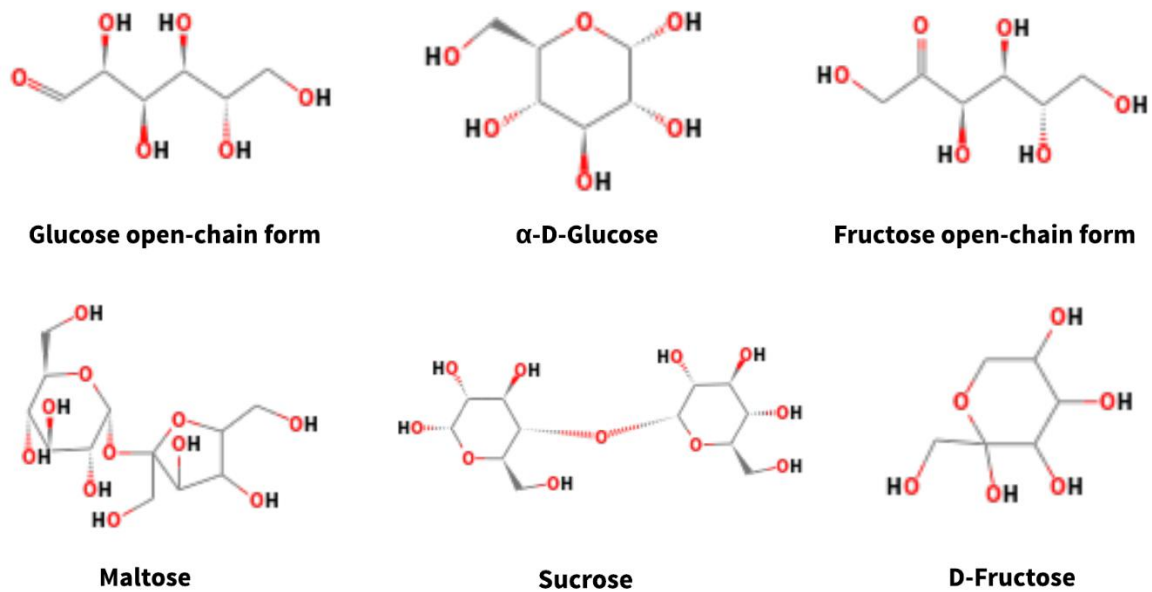


Figure 2 - Example of some reducing sugars (glucose, fructose, and maltose) and non-reducing sugar structure (sucrose), adapted from ref. (38)

### 2.3 Differences between ethanol industry from corn and sugarcane

In general, ethanol industrial processes vary according to the feedstock applied. Nevertheless, the ethanol factories usually present two major steps: upstream and downstream.

In the upstream step, unit operations are used to obtain the fermentable sugar solution and carry out the fermentation of the sugar solution by the yeast. The subsequent step is called downstream, and it represents the set of unit operations used for the ethanol separation and purification by distillation-rectification-dehydration<sup>35</sup>.

As the bioethanol-producing process from sugarcane does not require a pre-treatment it is considerably feasible than bioethanol from starch<sup>32</sup>. In addition, sugarcane is more efficient than corn ethanol in its use of land once it can produce more than 45% ethanol per unit of land than corn<sup>39</sup>. Sugarcane has an ethanol production of 8,000L.ha<sup>-1</sup> (first harvesting) which is higher than corn ethanol production of 3,000L.ha<sup>-1</sup> <sup>34</sup>.

Ethanol from sugarcane presents lower costs than ethanol from corn, being R\$1.23.L<sup>-1</sup> for corn and R\$1.13.L<sup>-1</sup> for sugarcane. On the other hand, a ton of corn can produce 407L of ethanol while a ton of sugarcane can produce only 89.5L of ethanol<sup>40</sup>.

The main characteristics of the ethanol production process from the most used feedstocks in the world: corn and sugarcane are described in Table 2.

Table 2 - Main characteristics of ethanol industrial process from corn and sugarcane, adapted from ref. (14)

<b>Characteristics</b>	<b>Corn</b>	<b>Sugarcane</b>
<b>Fermentation process</b>	No yeast recycling	With yeast recycling
<b>Suspended solids</b>	>30%	<1%
<b>Ethanol yield</b>	85-90%	90-92%
<b>Fermentation time</b>	54-72 hours	6-12 hours
<b>Yeast concentration</b>	3-4%	8-12%
<b>Ethanol content</b>	12-18% (v.v <sup>-1</sup> )	7-12%
<b>Factory operation time</b>	345 days/year	200-240 days/year
<b>Main subproduct</b>	DDGS for animal feed	Stillage for fertigation

Ethanol production from corn also offers advantages in terms of its storage. Corn grains can be stored up to 3 years, however, sugarcane must be crushed until 72 hours after the harvest, to avoid sugar losses due microbial contamination<sup>35,41</sup>. Consequently, ethanol corn factories operate their fermentation processes during more days per year than ethanol sugarcane factories.

## 2.4 Ethanol industrial process from corn

Among the starchy crops, corn is widely used to produce ethanol. Corn main components (w.w<sup>-1</sup>) are 71% starch, 12% proteins, 8% lipids, 6% ash and 4% water<sup>42</sup>.

The starch fraction from corn is the component used to produce first-generation ethanol. However, before the fermentation processes, the starch needs to be cooked and hydrolyzed by enzymes like alpha-amylase and amyloglucosidase<sup>35</sup>. By this saccharification process, the starch is converted into reducing sugars as glucose, and maltose to become available for yeast fermentation.

The dry milling flow diagram for ethanol-producing and its co-products is shown in Figure 3.



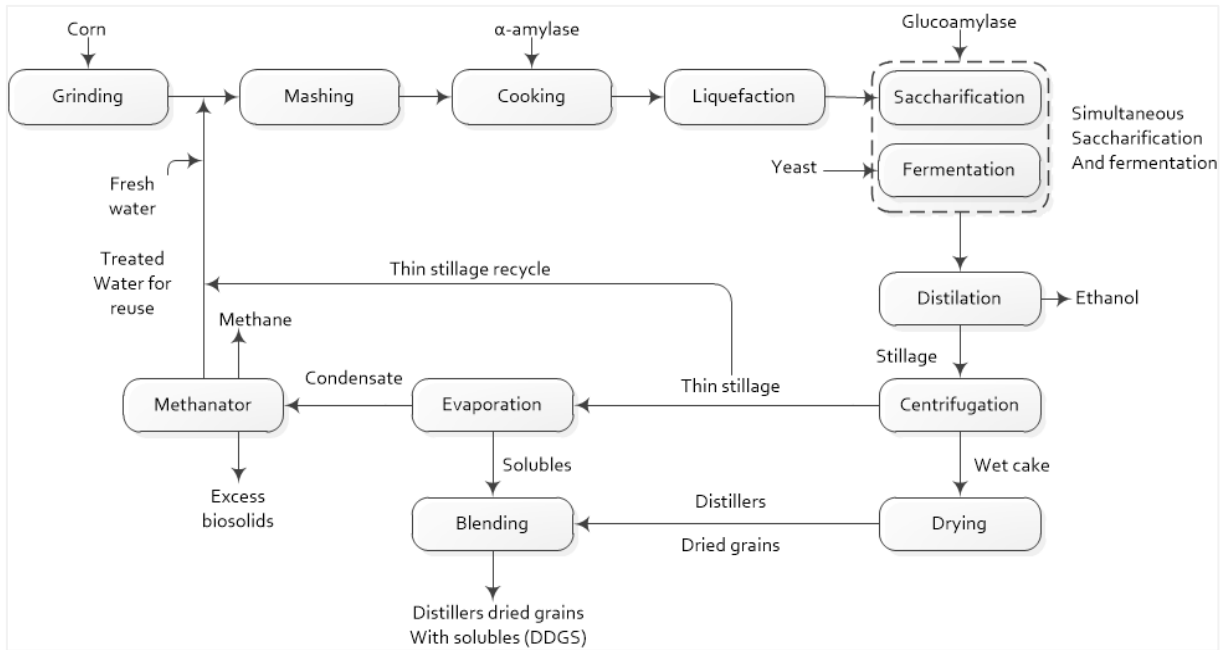


Figure 3 - Ethanol production from dry milling process<sup>35</sup>

In the ethanol industry from corn there are two major processes: the dry milling and the wet milling. Dry milling is more widely used to produce ethanol than wet milling, which can also produce other high-value products such as corn syrup and dextrose<sup>43</sup>.

In the dry milling process the water is added after the corn has been milled. Moreover, the corn hydrolysis and fermentation are conducted into the same batch, which is called simultaneous saccharification and fermentation (SSF) processes. Usually, SSF is used to reduce costs related with saccharification batch acquisition and to reduce contamination risks<sup>35</sup>. By carrying out the hydrolysis and fermentation in the same batch, there is less need to transfer the hydrolysate to a separate vessel for fermentation, which can reduce the risk of contamination and simplify the process. Additionally, by using the same batch for both processes, the costs associated with acquiring separate vessels for hydrolysis and fermentation are eliminated.

In the wet milling process the corn is milled in an aqueous solution and it is divided into three components: the hull, the germ, and the endosperm. From the endosperm component, it is possible to obtain the starch after the degemination step. Once the starch has already been hydrolyzed and the saccharification step is over, the yeast is added, and the fermentation process is carried out during 20 to 60 hours<sup>43</sup>.

The wet milling flow diagram for ethanol-producing and its co-products is shown in Figure 4.

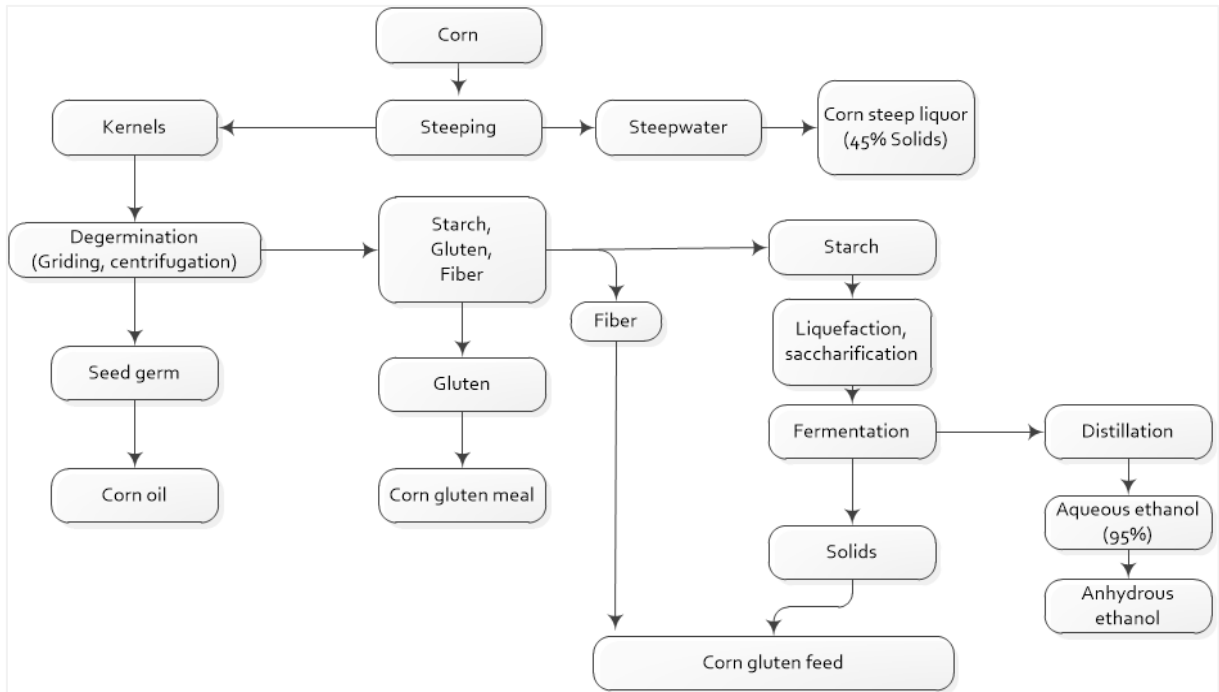


Figure 4- Ethanol production from wet milling process<sup>35</sup>

As mentioned above, in the wet milling process the saccharification and fermentation steps are commonly conducted in separated steps. Besides that, in this operation less insoluble solids are found which allows the yeast recycling after the fermentation<sup>35</sup>.

## 2.5 Ethanol industrial process from sugarcane

Sugarcane is a semi-perennial crop originating in Southeast Asia and it is used in bioethanol production. This feedstock is composed of 84-90% broth and 10-16% fibers containing cellulose, hemicellulose, and lignin. Its broth is formed of a solution with 18-25% soluble solids and 75%-82% water. Sucrose represents almost 17% of sugarcane soluble solids while fructose and glucose represent 1%<sup>44</sup>.

When using sugarcane, the yeast can directly assimilate glucose and fructose. In the case of sucrose, the yeast needs to hydrolyze it by invertase enzyme and then can easily assimilate it<sup>34</sup>.

In Brazil, sugarcane distilleries operate in a process called Melle-Boinot, being 75% as fed-batch and 25% in continuous mode. Melle-Boinot process was developed and patented by Firmin Boinot from Melle region (France) in 1930 years. The main aspect of the patent was the centrifugation and recycling of yeast cells, acid treatment,

high cell density and faster fermentations than without recycling processes. Continuous fermentation with recycling of yeast cells was a variation from Melle-Boinot<sup>13</sup>. The flow diagram for ethanol-producing from sugarcane by Melle-Boinot process (Fed-batch) is shown in Figure 5.

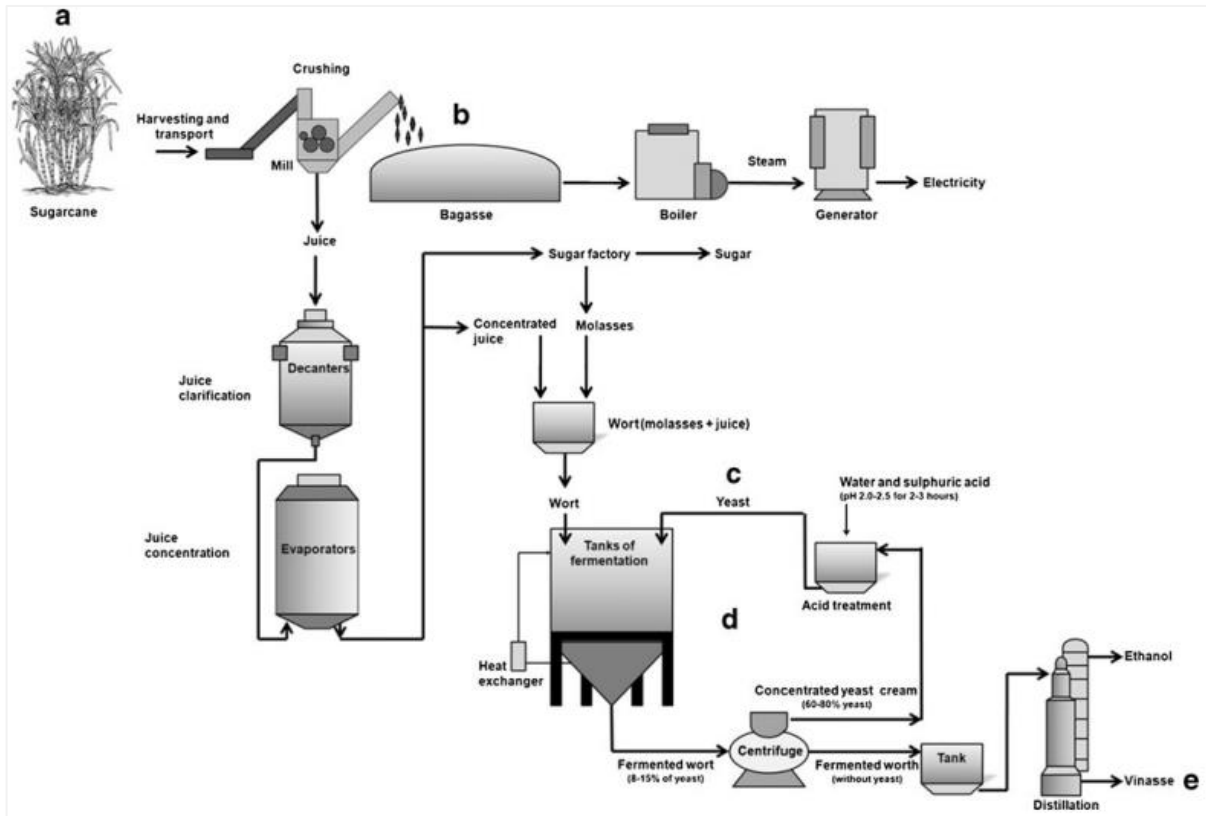


Figure 5- Ethanol production from sugarcane by Melle-Boinot process (fed-batch mode)<sup>13</sup>

Both processes, fed-batch or continuous mode, employ yeast cell recycling (90-95%) and they also perform fermentations with yeast in high density into fermenters, up to 10-14% wet weight/volume. For these reasons, sugarcane fermentation takes less time (6-10 hours) than corn fermentation (54-72 hours) due its yeast high density into fermenters. In addition, these sugarcane fermentation characteristics contribute to driving sugar for ethanol production instead of deviating it for biomass formation<sup>34,45</sup>.

After the sugarcane fermentation the wine containing yeast is centrifuged. While the clarified wine goes to the distillation, the yeast cream is submitted to an acid treatment. Into the yeast treatment tank, the yeast cream is diluted with water, and sulfuric acid is added to reach a pH between 2.0 to 2.5 for 1 to 2 hours. Then, it can be used in the next fed-batch fermentation cycle<sup>46</sup>.

## 2.6 Ethanol production process from corn and sugarcane using only a fermentation line

Although most of the ethanol produced in Brazil comes from sugarcane, corn ethanol has been gaining ground in the market. According to the Brazilian Sugarcane Industry Association<sup>47</sup>, ethanol from corn reached 2.57 billion liters in the 2020/2021 harvest, which means an increase of 58.13% compared to the 2019/2020 harvest. Furthermore, it represented 8.45% of the biofuel total production in the Brazil Center-South<sup>47</sup>.

Regarding this, there are already in Brazil flex factories producing ethanol from corn during sugarcane off season. Although an industrial production using only a fermentation line for both feedstocks still require further investments<sup>47</sup>.

The cost of ethanol in the United States and Brazil is influenced by the current exchange rate and the prices of raw materials<sup>39</sup>. Any changes in these factors can have a significant impact on the relative cost of ethanol production and pricing in both countries. In that sense, mixing the feedstocks used for ethanol production is a way to keep the bioethanol competitive for domestic consumption or exports.

Although corn is the primary source of bioethanol globally, the corn ethanol industry in the United States relies heavily on the fossil fuels burning during production. This practice is widely regarded as less sustainable compared to ethanol production from sugarcane. In contrast, in Brazil, distilleries have opted to use sugarcane bagasse or wood chips instead of fossil fuels for ethanol production.

The sugarcane juice addition in a corn ethanol plant provides sugars and nutrients for yeast metabolism<sup>9</sup>. Consequently, the corn amount and enzymes required in the process can be reduced by up to 50%. The authors also state that this integration reduces the water amount used to prepare the wort. Thus, it can increase the efficiency of alcoholic fermentation by around 4.4%<sup>9</sup>.

It is noteworthy that the energy generation by burning sugarcane bagasse reduces the fossil fuels burning by the corn ethanol industry<sup>48</sup>. This aspect increases the energy balance and sustainability of the corn ethanol industry.

As the sugarcane juice also carries a microbial load in its content, further research about mixed wort decontamination is required before integrating an ethanol plant from corn and sugarcane using just a fermentation line.

## 2.7 Factors affecting industrial alcoholic fermentation

Alcoholic fermentation quality relies on setting parameters such as temperature, pH, nutrients, total reducing sugars, agitation, and oxygen at the recommended levels. The optimal pH for *Saccharomyces cerevisiae* cultivation is around 4.0-5.0 while the temperature is about 30°C<sup>49</sup>.

Nutrients like Nitrogen, Phosphorus, Potassium, Magnesium, Calcium, Zinc, Manganese, Cobalt, Iron, Copper, and Sodium also play important roles in the yeast metabolism<sup>10</sup>. The total reducing sugars - TRS act as carbon sources for the yeast. However, when it is in the medium in concentrations higher than ca. 150g.L<sup>-1</sup> it can inhibit the *Saccharomyces cerevisiae* growth<sup>50</sup>.

The stress factors upon yeast during industrial fermentation and its response by producing substances such as trehalose, glycogen, glycerol, and succinic acid are shown in Figure 6<sup>14,51</sup>.

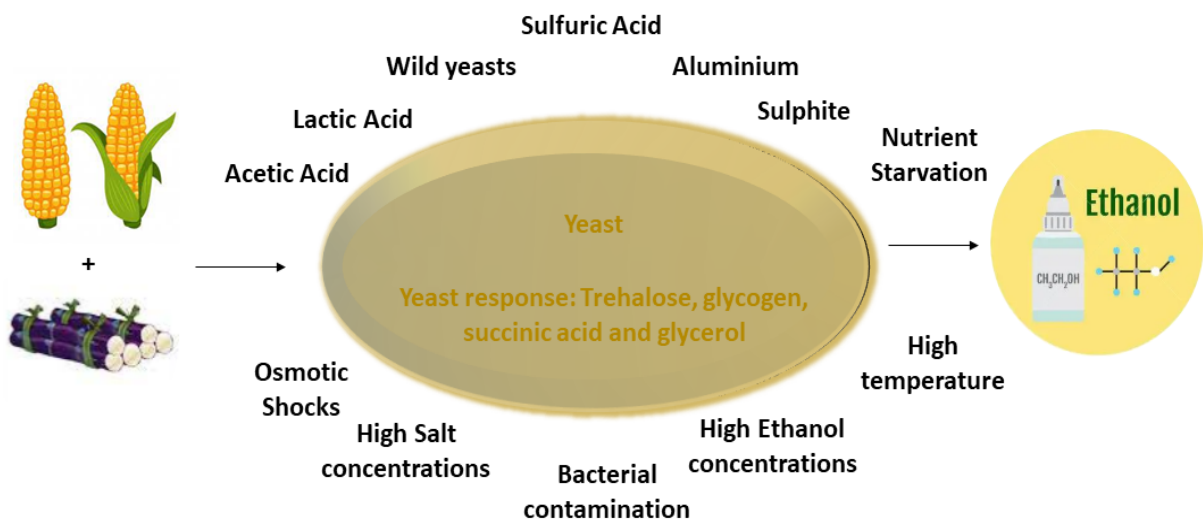


Figure 6 - Scheme of the stressing factors upon yeast during industrial fermentation and its metabolism response by glycerol, succinic acid, glycogen, and trehalose production, adapted from ref. (14)

During industrial fermentation the yeast is exposed to unfavourable conditions, which can interfere in its performance in converting sugar to ethanol. In Brazilian distillers the yeast face unfavourable conditions like high ethanol concentrations, sulfuric acids, high concentration of salts, aluminium, osmotic stress, nutrient starvation, lactic acid, acetic acid, bacterial contamination, wild yeasts, high temperatures and sulphite<sup>14,51</sup>.

### 2.7.1 Microbial contamination

The bacterial contaminant loads in wet milling and dry milling corn ethanol plants are approximately,  $10^6$ CFU.mL<sup>-1</sup> and  $10^8$ CFU.mL<sup>-1</sup>, respectively<sup>11</sup>. The survey carried out by Skinner and Leathers<sup>11</sup> showed that 77% of bacteria isolated from corn-based ethanol plants were from the *Lactobacillus* genus. In addition, genus such as *Bifidobacterium*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Weisella* were also isolated from the wort<sup>11</sup>.

In accordance with the results found by Skinner and Leathers<sup>11</sup>, Bischoff<sup>52</sup> reported that 50% of the isolated microorganisms from corn wet milling and dry milling plants were from the genus *Lactobacillus*.

Queiroz<sup>12</sup> evaluated the microorganism selection arising in sugarcane ethanol plants in Brazil. According to their findings, *Lactobacillus* was the genus present in all fermentation steps<sup>12</sup>. This genus represented more than 77.95% of the microbial communities in all fermentation steps, followed by *Acinetobacter* and *Zymomonas*<sup>12</sup>. The temperature was the selection factor with more impacts on the appearance of new species during sugarcane ethanol fermentation<sup>12</sup>.

Gram-positive bacteria are the major bacterial contamination during alcoholic fermentation, mainly due to *Lactobacillus* activity<sup>45</sup>. A lactic acid bacterial load of about  $6.0 \times 10^5$ - $8.9 \times 10^8$  CFU.mL<sup>-1</sup> was evaluated in sugarcane ethanol mills in Brazil<sup>53</sup>. However, reduction on ethanol yields also has been reported due to the gram-negative bacteria identified as *Acetobacter pasteurianus*<sup>45</sup>.

As lactic acid bacteria show tolerance to alcohol, fast growth and it is adaptive to low pH mediums, they can dominate the process and inhibit the yeast growth<sup>54</sup>. Consequently, it reduces the yeast viability, process productivity and ethanol yield<sup>11,55</sup>.

## 2.8 Decontamination methods for alcoholic fermentations and electron beam applications

On an industrial scale, sugars fermentation present in sugarcane and corn are widely used for bioethanol production<sup>56</sup>. However, during the wort fermentation, *Bacillus* and *Lactobacillus* bacteria excrete organic acids and toxins in the medium. Consequently, it reduces the ethanol yield and increases the acidity in the fermented wort<sup>14</sup>.

Conventionally, antibiotics, concentrated sulphur compounds and biocides are used to contain microbial contamination, however, these substances increase ethanol cost<sup>57</sup>. When concentrated sulphur compounds are not enough to control the microbial contaminants load, antibiotics are added in the process<sup>16</sup>. Despite the antibiotic's efficiency, concerns are being raised about their use. One of them regarding the antibiotic retention in dry yeast marked as livestock feed. Another one is about the evidence of resistant bacterial appearance, which increases the required antibiotic doses to control the bacterial activity<sup>58</sup>.

Studies have been carried out to replace antibiotics for alternative antimicrobial substances. Natural products from fruits, animals, seeds, and plants contain bacteriostatic and bactericidal properties and it can inhibit microbial contaminants<sup>59</sup>. Between the plants hop is the most used to inhibit contaminants in breweries<sup>16,60</sup>. Propolis from honeybees and chitosan extracted from the chitin of crustacean shells are animal-derived substances with antimicrobial properties<sup>61,62</sup>.

Microorganisms have different relationships between them since symbiosis to competition. In that order, microorganisms can also be used to control bacteria activity during alcoholic fermentations<sup>16</sup>. Bacteriophages can inhibit bacterial strain *L. fermentum* in corn mash<sup>62</sup>. At the end of the fermentation carried out with bacteriophage's addition, the author observed a reduction in the organic acid and a restoration in the ethanol yield.

On an industrial scale, only chlorine dioxide solution at 30mg.L<sup>-1</sup> represents an alternative substance to replace antibiotics and sulfuric acid treatment<sup>63</sup>.

In addition to those decontamination methods discussed previously, the electron beam irradiation can be applied to eliminate microorganisms in wort for fermentations<sup>15</sup>. The technique is easy to implement in a factory line and can also be produced in sugarcane ethanol plants by its energy surplus<sup>15</sup>. In that order, researchers should be carried out to verify the technical and financial aspects of implementing electron beam irradiation in an industrial process to produce ethanol.

Electron beam irradiation is produced in equipment called electron accelerators. In this equipment, a high-voltage potential is established between the cathode and anode in the evacuated tube<sup>65</sup>. The electrons are emitted and accelerated to high velocities in an electron gun, usually containing a cathode, grid, and anode. These accelerated electron beams emerge from the gun and when in touch with a material, they can eliminate unwanted microorganisms<sup>65</sup>. An electron beam accelerator scheme

including the following components: electron gun, magnetic focusing lens, grid, anode, magnetic deflection coil and cathodic emitter is shown in Figure 7<sup>65</sup>.

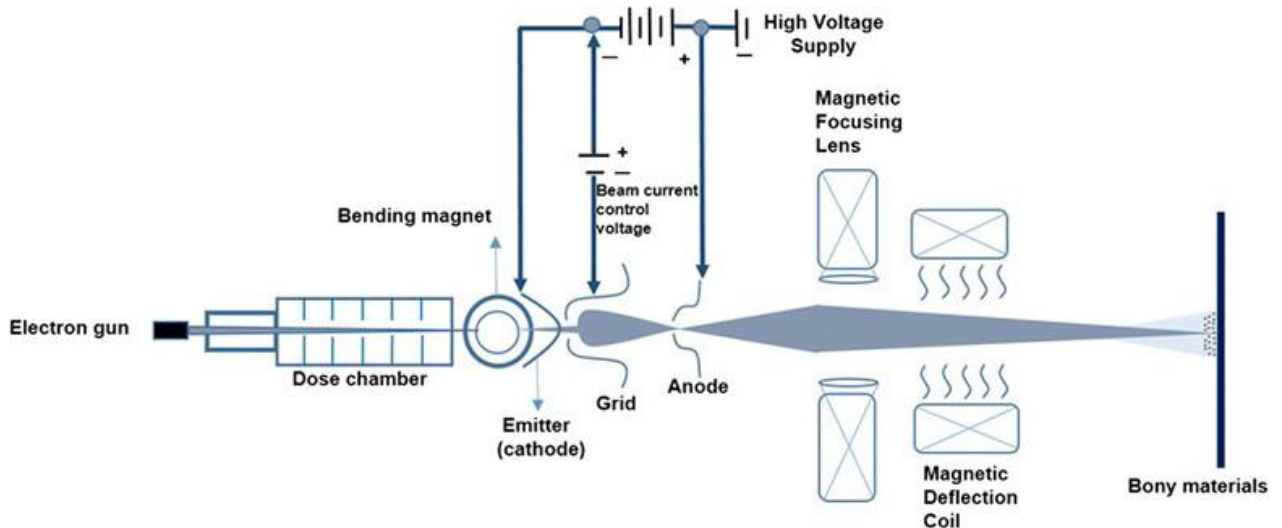


Figure 7- Schematic drawing of an electron beam accelerator<sup>65</sup>

The high energy and the ability to penetrate materials of the electron beam produced in accelerators are crucial properties that are explored when ionizing radiation is applied. Within the food industry, the electron beam is primarily utilized for the elimination or sterilization of pests and pathogens from agricultural products, resulting in improved shelf life and safety<sup>21</sup>. Additionally, the electron beam technique is applied in the enzyme inactivation process within the fruit juice industry, resulting in agribusiness improvements by extending product shelf life and maintaining its quality<sup>21,66</sup>. In this context, during food irradiation, microorganisms are eradicated through DNA damage, rendering any attempt at replication unfeasible<sup>67</sup>.

In the beverage industry, potential applications of electron beam relate to the sterilization of wort, packaging materials such as bottles, cans, and processing and storage containers<sup>19</sup>.

Despite the use of electron beam to sterilize liquids and materials, the first major applications of this technique were to modify material and produce value added products like heat-resistant wires, foamed plastics, automotive tires, semiconductors, and electronic components<sup>19</sup>. The main processing technology for industrial and environmental application of electron beam accelerators in Latin America and Caribbean are shown in Table 3<sup>68</sup>.



Table 3 - Industrial electron beam accelerator application in Latin America and the Caribbean<sup>68</sup>

<b>Country</b>	<b>City</b>	<b>Products</b>
Brazil	São Paulo	Wastewater treatment, polymer modification, shrink tube and film, surface curing, food irradiation, wire and electric cables, semiconductors, sterilization of medical and pharmaceutical devices, and PE foam sheets
Ecuador	Quito	Food irradiation, wires, and electric cables
Mexico	Tijuana, Ensenada, Mexico City	Polymer modifications (plastics and rubber), sterilization of medical devices, polymer modifications, and fresh food packaging
Costa Rica	Alajuela	Sterilization of medical and pharmaceutical devices
Dominican Republic	Haina	Sterilization of medical and pharmaceutical devices

Notably, electron beams have a wide potential of applications, spanning from sterilization of medical care products and materials science to the energy and food industries. Given the ongoing research and development in this field, the outlook for this technology utilization is a growing in both terms of economic scale and in the identification of novel applications<sup>19</sup>.

### **3. OBJECTIVES**

The primary objective of this study is to investigate the parameters associated with ethanol production from corn and sugarcane mixed wort irradiated by electron beam. In that order, the main parameters examined to assess alcoholic fermentation include ethanol yield (both technological and stoichiometric), alcohol content, productivity, yeast-specific growth rate, cell viability, total reducing sugars (TRS), and the concentrations of substances produced during fermentation, such as glycerol, organic acids, and mannitol.

Furthermore, this work aims to conduct a physicochemical characterization of both feedstocks and examine the impact of nutrient supplementation on corn and sugarcane mixed wort within a single fermentation process.

## 4 MATERIALS AND METHODS

To provide a systematic view, the four topics studied in this work were divided into three groups: **inputs** (the conditions adopted for each topic), **main activities** (the experiments conducted), and **outputs** (the findings or response variables derived from the experiments). An illustrative representation is shown in Figure 8.

Methodology Flowchart				
Topic	Process	Input (Condition)	Main activities	Output
Topic I	Feedstock physicochemical characterization	<div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Corn hydrolysate</div> <div style="border: 1px solid black; padding: 2px;">Sugarcane syrup</div>	<div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Feedstock preparation</div> <div style="border: 1px solid black; padding: 2px;">Digestion by wet oxidation</div>	<div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Carbohydrate content (Glucose, fructose, sucrose, and maltose)</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Density, and pH</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Carbon and nitrogen content</div> <div style="border: 1px solid black; padding: 2px;">Content of other nutrients: K, P, Mg, Na, Ca, Zn, Fe, Cu, Co, and Mn</div>
Topic II	Assessment of nutrients' supplementation impacts on alcoholic fermentation	<div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Control (Mixed wort, 80% corn hydrolysate, and 20% sugarcane syrup, without any decontamination treatment or supplementation)</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Control + Calcium</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Control + Manganese</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Control + Magnesium</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Control + Sodium</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Control + Potassium</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Control + Phosphorus</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Control + Zinc</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Control + Copper</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Control + Iron</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Control + Cobalt</div> <div style="border: 1px solid black; padding: 2px;">Control + Nitrogen</div>	<div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Fermentation in microplate for ELISA</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Fermentation in conical tubes</div> <div style="border: 1px solid black; padding: 2px;">Nutrient solution preparation</div>	<div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Ethanol Red growth profile</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Yeast specific growth rate</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Concentration of total reducing sugars (TRS), ethanol, glycerol, organic acids, and mannitol</div> <div style="border: 1px solid black; padding: 2px;">Fermentative parameters (Ethanol yield, productivity, and alcohol content)</div>
Topic III	Effects of electron beam and antibiotic on wort decontamination	<div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Control (Mixed wort, 80% corn hydrolysate, and 20% sugarcane syrup, without any decontamination treatment or supplementation)</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Control treated by e-beam at 10 kGy</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Control treated by e-beam at 15 kGy</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Control treated by e-beam at 20 kGy</div> <div style="border: 1px solid black; padding: 2px;">Control treated by antibiotic (commercial name: Kamoran HJ; active ingredient: sodium monensin crystalline 80%)</div>	<div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Assessment of electron beam treatment on carbohydrate content</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Evaluation of electron beam and antibiotic treatment on wort decontamination</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Fermentation in microplate for ELISA</div> <div style="border: 1px solid black; padding: 2px;">Fermentation in conical tubes</div>	<div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Ethanol Red growth profile</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Yeast specific growth rate</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Concentration of total reducing sugars (TRS), ethanol, glycerol, organic acids, and mannitol</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Fermentative parameters (Ethanol yield, productivity, and alcohol content)</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Cell viability</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Total contamination</div> <div style="border: 1px solid black; padding: 2px;">Bacterial contamination</div>
Topic IV	Impacts of scaling up mixed wort fermentation	<div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Control (Mixed wort, 80% corn hydrolysate, and 20% sugarcane syrup, without any decontamination treatment or supplementation)</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Control treated by e-beam at 20 kGy</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Control treated by e-beam at 20 kGy + supplementation (Nitrogen, Manganese, Potassium, and Cobalt)</div> <div style="border: 1px solid black; padding: 2px;">Control treated by antibiotic (commercial name: Kamoran HJ; active ingredient: sodium monensin crystalline 80%)</div>	<div style="border: 1px solid black; padding: 2px;">Fermentation in benchtop bioreactor</div>	<div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Cell viability</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Bacterial contamination</div> <div style="border: 1px solid black; padding: 2px; margin-bottom: 2px;">Concentration of total reducing sugars (TRS), ethanol, glycerol, organic acids, and mannitol</div> <div style="border: 1px solid black; padding: 2px;">pH</div>

Figure 8- Description of the dissertation methodology

## 4.1 Feedstocks

The feedstocks used to prepare the mixed wort were corn hydrolysate and sugarcane syrup. Both raw materials were provided by the Laboratory of Sucrenergetic and Bioenergy Technology (LTSBio) - ESALQ/USP located in Piracicaba/Brazil. Corn hydrolysate and sugarcane syrup were stored at - 4°C until used, as performed by reference<sup>69</sup>.

## 4.2 Topic I: Feedstock physicochemical characterization

Some nutrients, such as Nitrogen and Phosphorus improve yeast metabolism through its action and increase ethanol yield. However, when the parameters exceed yeast requirements, they can play a role in inhibiting yeast growth. For this reason, before wort preparation, corn hydrolysate and sugarcane syrup were characterized regarding its carbohydrate content (glucose, fructose, sucrose, and maltose), pH, density, and the following nutrients: Carbon, Nitrogen, Calcium, Magnesium, Manganese, Sodium, Potassium, Phosphorus, Zinc, Copper, Iron and Cobalt as described in Table 4.

Table 4 - Feedstock physicochemical characterization

Analyses	Method	Reference
Total organic carbon -TOC Inorganic carbon - IC Total carbon - TC Total Nitrogen - TN Carbon/Nitrogen - C/N	Catalytic combustion at high temperature (total organic carbon analyzer TOC-L CPH / CPN analyzer)	Ref. (50)
Potassium, Phosphorus, Magnesium, Sodium, Calcium, Manganese, Zinc, Iron, Cobalt, and Copper	Inductively coupled plasma optical emission spectrometry - (ICP OES)	Ref. (70)
Total reducing sugars (Fructose, glucose, sucrose, and maltose)	Ion chromatography	Ref. (71,72)
pH	Hydrogen potential	Ref. (50)
Density	Hydrometer	Ref. (73)

To determine carbon, and nitrogen in TOC, and the other nutrients (Calcium, Magnesium, Manganese, Sodium, Potassium, Phosphorus, Zinc, Copper, Iron, and

Cobalt) in ICP-EOS the samples from the feedstocks were digested by wet oxidation. The carbohydrates (glucose, fructose, sucrose, and maltose) were analyzed by ion chromatography 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  $\mu\text{m}$  and analyzed.

### 4.3 Wort preparation

The wort was prepared considering a hybrid feeding system composed of 80% v.v<sup>-1</sup> corn hydrolysate and 20% v.v<sup>-1</sup> sugarcane syrup. Before mixing the material, corn hydrolysate was filtered through 0.75 $\mu\text{m}$  sieves to avoid solids in the wort. Then, both feedstocks were diluted with distilled water to obtain a TRS concentration of approximately 120g.L<sup>-1</sup>. During wort preparation, the primary objective was to ensure that the concentration of TRS (total reducing sugars) remained below ca. 150g.L<sup>-1</sup> (15°BRIX), as concentrations higher than this may lead to substrate inhibition<sup>50</sup>. Lastly, they were mixed and kept at - 4°C until used.

The hybrid feeding system composed of corn hydrolysate and sugarcane syrup to prepare the mixed wort for alcoholic fermentation is exhibited in Figure 9.

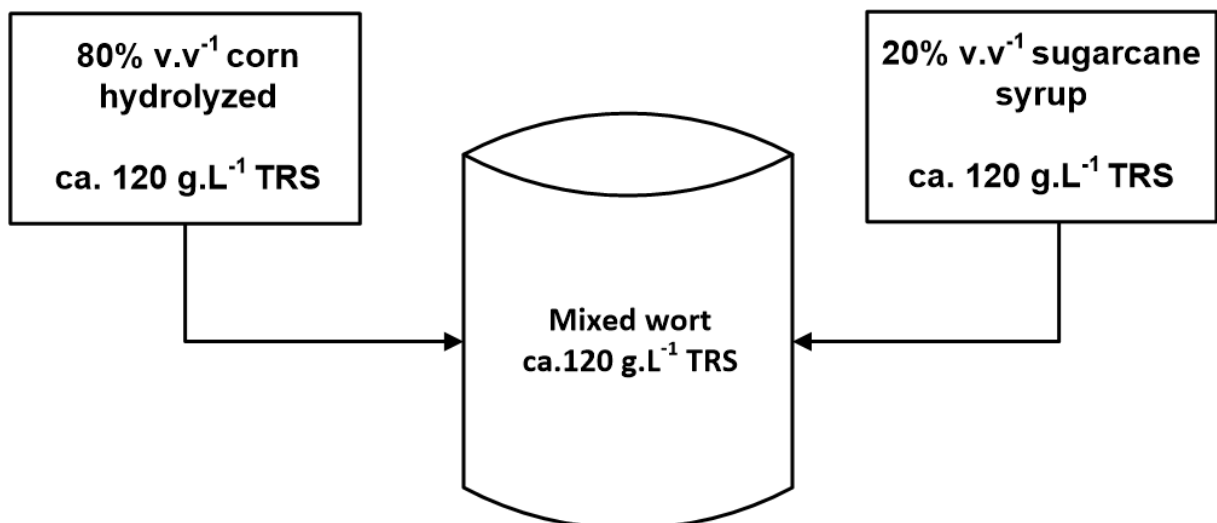


Figure 9- Mixed wort preparation for alcoholic fermentations

### 4.4 Inoculum preparation (yeast culture)

Ethanol Red™ was the *S. cerevisiae* strain used to conduct the fermentations. This strain was chosen because it was specially developed for the corn ethanol

industry and 80% of the mixed wort was from corn. The strain was kindly provided by the Bioprocess Engineering Laboratory - BELa from the University of São Paulo, Brazil. Ethanol Red stock was prepared and stored into cryogenic tubes at  $-80^{\circ}\text{C}$ . The stock was made into 1mL cryogenic tubes containing 80% v.v<sup>-1</sup> yeast cells in YPD broth and 20% v.v<sup>-1</sup> glycerol.

YPD broth was the culture medium used to prepare the pre-inoculum and the inoculum. It consisted of an aqueous solution with yeast extract 1% v.v<sup>-1</sup>, dextrose 2% v.v<sup>-1</sup> and peptone 2% v.v<sup>-1</sup>. Before adding 1mL of Ethanol Red to the pre-inoculum preparation, YPD broth was autoclaved at  $120^{\circ}\text{C}$  and  $1\text{kg}\cdot\text{cm}^{-2}$  for 30 minutes.

To ensure optimal yeast growth and activity, a pre-inoculum was first prepared by keeping yeast cultures overnight on a shaker at  $30^{\circ}\text{C}$  and 180rpm. Next, to enhance yeast adaptability, a 1mL aliquot was extracted from the pre-inoculum and added to YPD broth, resulting in an inoculum with a yeast concentration of approximately 0.2 absorbance. The inoculum was then incubated overnight on a shaker under the same conditions used for the pre-inoculum, allowing for robust yeast growth and preparation for fermentation.

#### **4.5 Topic II: Assessment of nutrients' supplementation on alcoholic fermentation**

Twelve conditions were evaluated in this subsection including a control assay and eleven nutrients supplemented. In addition, the fermentations were carried out in microplate for ELISA (Enzyme-Linked Immunosorbent Assay) and in conical tubes. A schematic diagram about the experiments carried out to assess the nutrients supplementation' impacts on alcoholic fermentation can be seen in Figure 10.

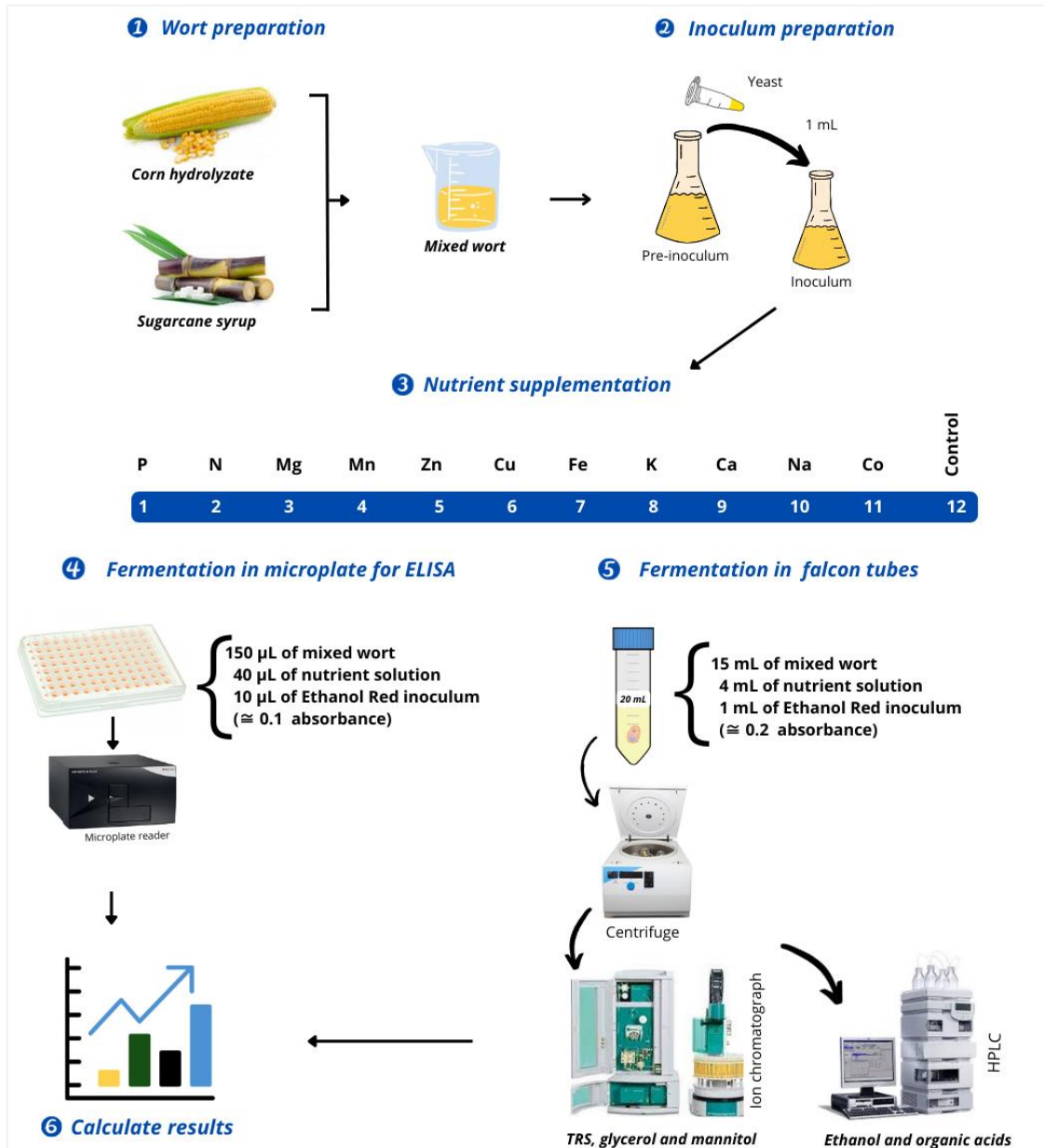


Figure 10 - Scheme of experiments carried out to assess the nutrients supplementation impact on alcoholic fermentation

Before starting, conical tubes were filled with 15mL of mixed wort and 4mL of nutrient solution or water for the control sample. In addition, 1mL of Ethanol Red inoculum was added, to start the fermentation with a yeast concentration of 0.2 absorbance and a total volume of 20mL. The TRS and metabolites were evaluated before and after alcoholic fermentations.

#### 4.5.1 Nutrient solution preparation

Since no studies about nutrients' supplementation in corn and sugarcane mixed wort was found, the concentration of each nutrient supplemented as well as its

chemical form supplied were defined by benchmarking the research with sugarcane wort performed by the company Fermentec and Santos<sup>74,75</sup>. All the information about the nutrients supplied, its chemical form and concentration to obtain adequate alcoholic fermentation are described in Table 5.

Table 5 - Conditions evaluated in the mixed wort supplementation tests<sup>74,75</sup>

Nutrient supplied	Chemical form	Nutrient concentration (mg.L <sup>-1</sup> )
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)	-

#### 4.6 Topic III: effects of electron beam and antibiotic on wort decontamination

During the ethanol production process corn hydrolysate and sugarcane syrup are contaminated by microorganisms, mainly gram-positive bacteria. These microorganisms compete with yeast when they metabolize sugars and excrete toxic substances in the wort. Consequently, the acidity increases and ethanol yield decrease<sup>76</sup>.

In that regard, wort decontamination means an important step before starting alcoholic fermentation to increase ethanol yield. In this set of experiments, the alcoholic fermentations were carried out with mixed wort submitted to five different



decontamination conditions: irradiated with electron beam at 10kGy, 15kGy and 20kGy, treated with the antibiotic Sodium monensin 80% (Kamoran HJ at 3ppm), and with no treatment.

The first step to start the test was to add 10mL of yeast inoculum into the conical tubes (1% of yeast in wet weight). Subsequently, the tubes were centrifuged at 4,000rpm for 5min and the supernatants were discarded. Afterwards, 21.25mL of mixed wort were added. All the conical tubes were filled with 3.75mL of water, except the Kamoran condition which was added 3.75mL of Kamoran solution, to achieve a concentration of 3ppm into the tube and a total volume of 25mL.

A schematic diagram about the experiments carried out to assess the nutrients supplementation' impacts on alcoholic fermentation can be seen in Figure 11.

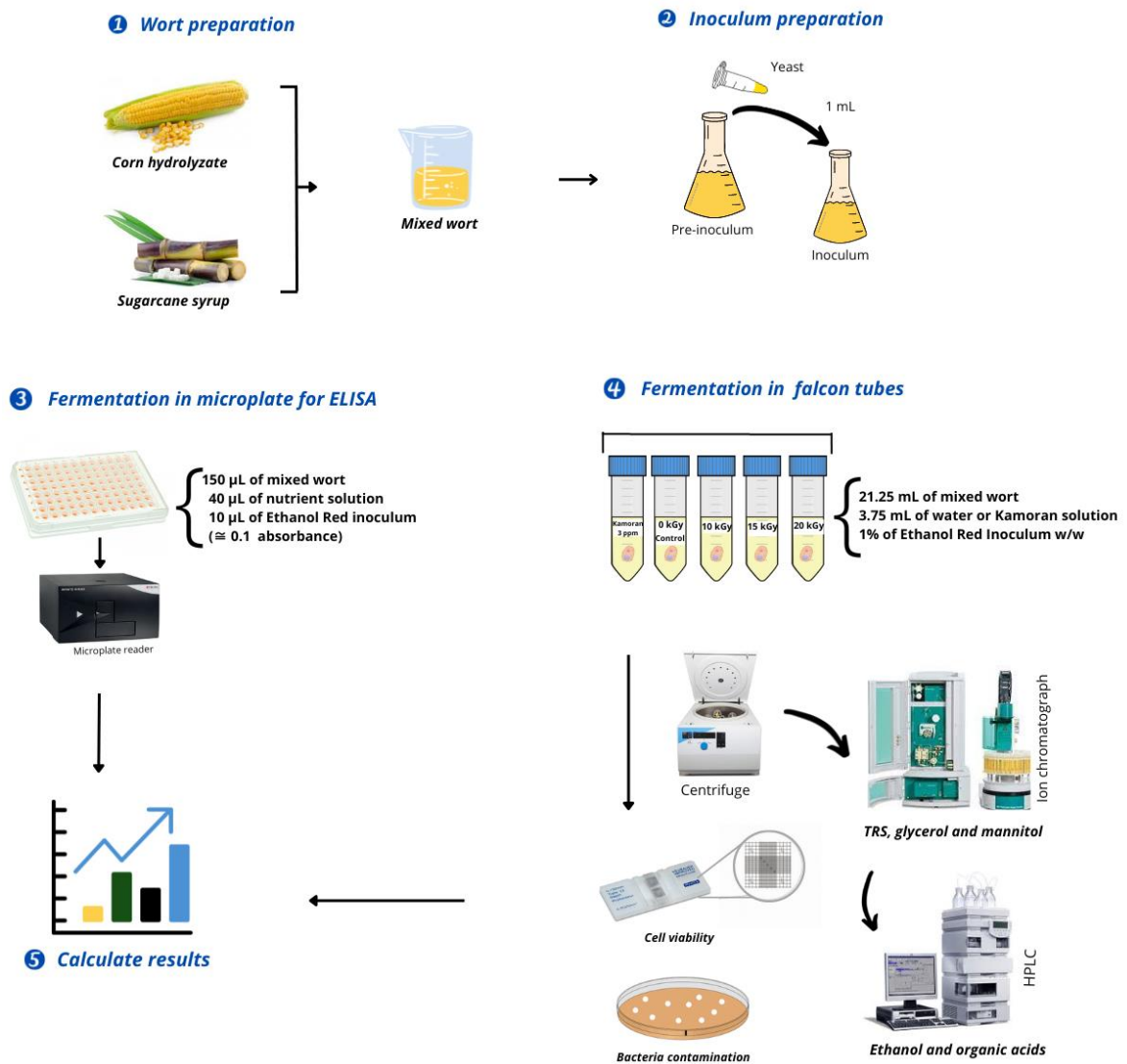


Figure 11 - Scheme of experiments carried out to assess the wort decontamination

#### 4.6.1 Antibiotic treatment

Antibiotic treatment experiments were carried out using the antibiotic Kamoran HJ which present Sodium monensin crystalline 80% as its active ingredient. The product was pre-diluted with a solution composed of 50% v.v<sup>-1</sup> water and 50% v.v<sup>-1</sup> alcohol as recommended by the supplier. The volume of antibiotic solution used in each fermentation was calculated in relation to the total volume of the wort, to reach 3ppm of antibiotic in the fermentation tanks.

#### 4.6.2 Electron beam treatment

A partnership with the Nuclear and Energy Research Institute - IPEN was made to conduct the electron beam irradiation treatment at the Radiation Technology Center - CTR/IPEN. The electron beam treatment was carried out using the Industrial Electron Accelerator Dynamitron - Job 188, model DC 1500/25/4, from the company Radiation Dynamics Incorporation - RDI ®. The processes for wort irradiation by Industrial Electron Accelerator Dynamitron can be seen in Figure 12.

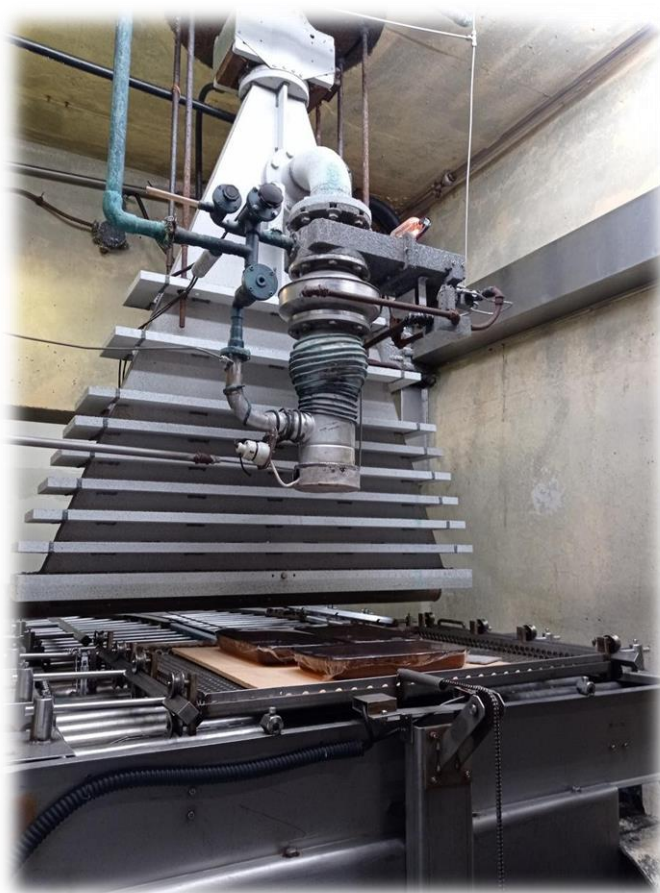


Figure 12 - Electron beam irradiation treatment

In this study, the radiation doses selected for the electron beam treatment were 0kGy, 10kGy, 15kGy and 20kGy. In addition, the samples were submitted for electron beam treatment in borosilicate glass containers (trays). The wort volume per tray was calculated to guarantee a 4mm height. Lastly, the trays were sealed with plastic film and placed on a 112cm conveyor belt with a speed of 6.72m.min<sup>-1</sup>.

As the liquid height in the trays varies with sample density, sample densities were measured using a densimeter (Table 4). The operating conditions for wort irradiation by the industrial electron accelerator dynamitron are shown in Table 6.

Table 6 - Operator conditions for wort irradiation by industrial electron accelerator dynamitron

<b>Total dose</b>	<b>0kGy</b>	<b>10kGy</b>	<b>15kGy</b>	<b>20kGy</b>
<b>Beam current (MeV)</b>	0	1.5	1.5	1.5
<b>Energy setup (mA)</b>	0	5.61	5.61	5.61
<b>Dose per pass (kGy)</b>	0	5	5	5
<b>Pass per Tray</b>	0	2	3	4

#### 4.7 Fermentations

Alcoholic fermentations were performed in microplates for ELISA, conical tubes, and benchtop bioreactor. Experiments in microplate for ELISA and in conical tubes were conducted to evaluate the best condition for alcoholic fermentation before beginning the scaling up to bioreactor.

Fermentations in microplates for ELISA were conducted in the Tecan Infinite® 200 PRO multimode plate reader using solid plates with 96 bottom wells. In addition, the wells were filled with the fermentation broth up to 200µL. In all fermentation in microplate for ELISA, 10µL of yeast inoculum was added (which meant an initial yeast concentration around 0.1 absorbance).

About the conditions settled, Ethanol's Red growth kinetics (output variable) were evaluated by measuring its absorbance at 600 nm at 30°C, every 20min for about 18 hours. The equipment orbital shaking amplitude and duration was defined in 1mm and 27s, respectively. Before starting the fermentation in microplate for ELISA, the

96 - well plates were sealed with sealing film to create a technical condition consider oxygen-limited or microaerobic.

Similarly, fermentations in conical tubes were also considered oxygen-limited or microaerobic. At this scale, the fermentations were carried out by monitoring the carbon dioxide loss. In that order, the tubes were not closed hermetically, and the masses of conical tubes were evaluated during the fermentation time on an analytical balance. Finally, the conical tubes were kept in a rotary shaker at 30°C, shaking at 180rpm, and the fermentation was stopped when variations in the conical tube's mass were less than 0.02g, up to 52 hours. The loss of water was not considered, and aliquots of 3mL were taken at the beginning and at the end of each fermentation.

The fermentation quality in conical tubes was evaluated by measuring the TRS (glucose, fructose, sucrose, and maltose) and the metabolites (ethanol, glycerol, mannitol, acetate, lactate, and succinate) before and after alcoholic fermentations.

Since the results interpretation are different for process with and without yeast recycling, it is noteworthy to state that all results in this work were obtained from batch fermentations without yeast recycling, in a non-simultaneous saccharification and fermentation process (SSF).

#### **4.7.1 Impacts of scaling up mixed wort fermentation**

Scale-up tests were conducted in a benchtop bioreactor Tec-Bio-Flex II from the company Tecnal, BR, with a total capacity of 1.5L, 45cm height, and 27cm width. In all fermentations, the bioreactor was fulfilled with 1,000mL of its total capacity (810mL of corn and sugarcane mixed wort, 40mL of the yeast inoculum (Ethanol Red 3% wet basis), and 150mL of distilled water or Kamoran HJ at 3ppm).

The bioreactor fermentations were conducted using a yeast concentration of 3%, mirroring the conditions found in corn ethanol factories. In contrast, fermentations in conical tubes and microplates for ELISA were performed with a lower yeast concentration of 1%. This allowed for the observation of a more well-defined yeast growth curve, encompassing lag, exponential, stationary, and decline phases. By varying the yeast concentration, we were able to examine the dynamics of the fermentation process more closely under the different scales and obtain a more comprehensive understanding of yeast behavior.

Finally, the temperature in the benchtop bioreactors was tightly controlled at 30°C using both a heating blanket and a thermostatic bath. Motor agitation was maintained at a constant speed of 180rpm. The experimental conditions and parameters are described in Figure 13, providing a clear overview of the approach used.



Figure 13 - Fermentation of corn and sugarcane mixed wort in bioreactor

In this topic, four conditions were selected to be tested on the bioreactors scale: mixed wort without treatment (control condition), treated by electron beam at 20kGy, Kamoran HJ at 3ppm, and mixed wort integrating supplementation and e-beam treatment at 20kGy. The nutrients selected for the bioreactor tests were Nitrogen, Manganese, and Potassium, as they demonstrated an increase in the fermentative parameters, and in the yeast specific growth rate. Furthermore, based on preliminary analyses, Cobalt was also supplemented in the wort for this test.

The alcoholic fermentation was monitored for 72 hours. During fermentation, aliquots of 5mL were taken at 0, 6, 24, 43, 54 and 72 hours. Cell viability, pH, and bacteria contamination in the Petri dish were the analyses carried out in the

experiments. In addition, aliquots were taken during the experiment to be analysed by ion chromatography, to quantify ethanol, organic acids, mannitol, and carbohydrates. The experimental design to evaluate the impacts of scaling up mixed wort fermentation in benchtop bioreactor is described in Figure 14.

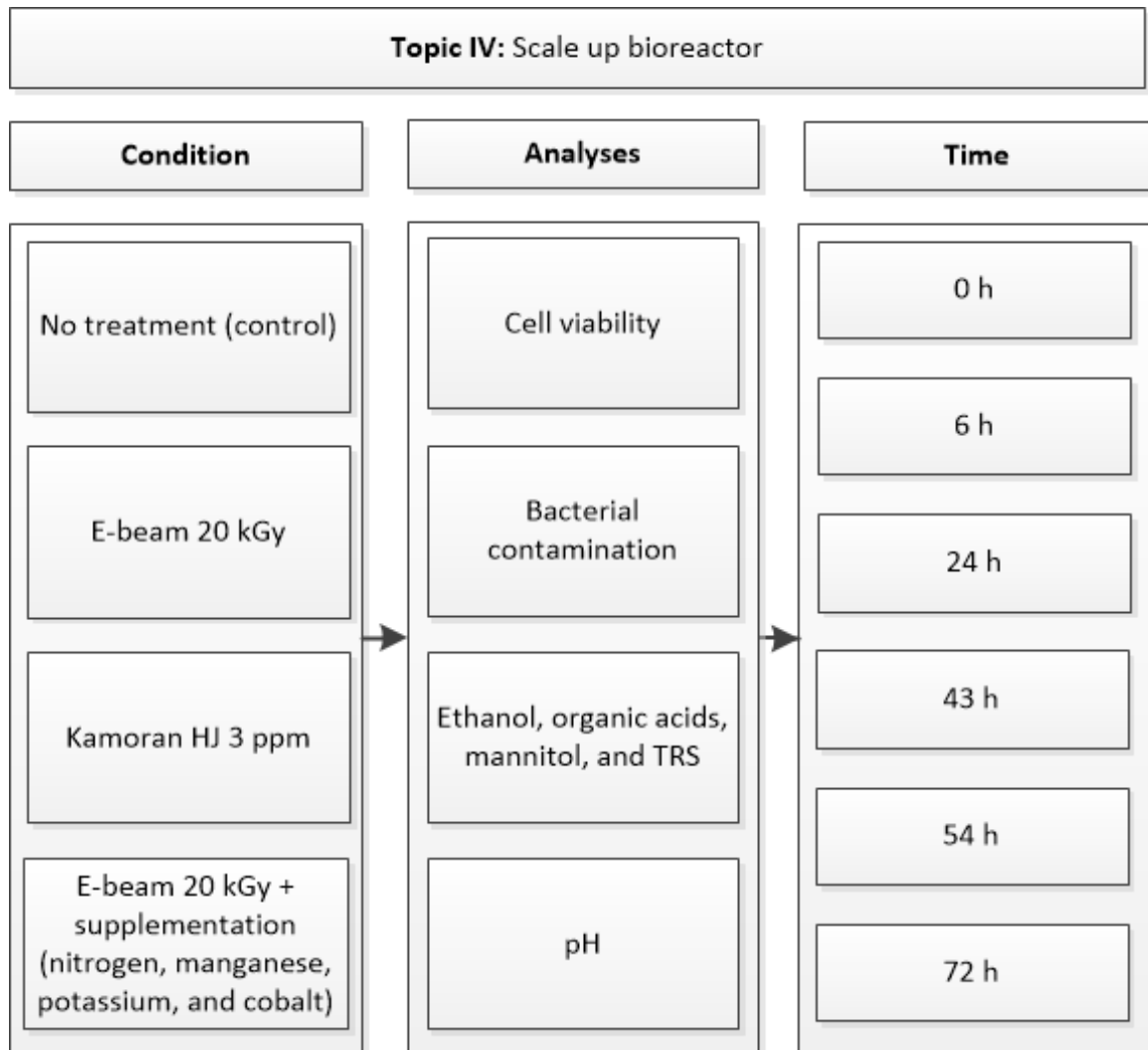


Figure 14 - Experimental design to evaluate the impacts of scaling up mixed wort fermentation

#### 4.8 Analyses (physicochemical, microbiological, and fermentative parameters)

In addition to the substrate characterization analyses in Table 4, analyses presented in Table 7 were performed before and after wort decontamination to select a decontamination method for the fermentation line scale-up.

Table 7 - Wort analyses before and after electron beam treatment

Analyses	Unit	Method	Reference
Total contamination	CFU.mL <sup>-1</sup>	Agar plate count	Ref. (77)
Bacteria contamination	CFU.mL <sup>-1</sup>	Agar plate count with cycloheximide	Ref. (77)
Total reducing sugar (Fructose, glucose sucrose and maltose)	g.L <sup>-1</sup>	Ion Chromatography	Ref. (78)

Alcoholic fermentation was evaluated using the analyses described in Table 8. The TRS and metabolites were measured at the end of each fermentation and during other fermentation stages.

Table 8 - Analyses to evaluate fermentation

Analyses	Unit	Method	Reference
Cell viability	%	Optical microscopy	Ref. (79)
Bacteria contamination	CFU.mL <sup>-1</sup>	Agar plate count	Ref. (78)
TRS and metabolites (ethanol organic acids, glycerol, and mannitol)	g.L <sup>-1</sup>	Ion Chromatography	Ref. (77)
Alcohol content (v/v)	%	$\frac{C[ethanol, f]}{789} \times 100$	Ref. (50)
Technological yield	%	$\frac{C[ethanol, f]}{0.511 \times C[TRS, i]} \times 100$	Ref. (50)
Stoichiometric yield	%	$\frac{C[ethanol, f - ethanol, i]}{0,511 \times C[TRS, i - TRS, f]} \times 100$	Ref. (50)
Ethanol productivity	g/L.h	$\frac{C[ethanol, f]}{t}$	Ref. (50)

Wherein:

$C[ethanol, f]$  = net concentration of ethanol in the fermented wort, g.L<sup>-1</sup>;

$C[ethanol, i]$  = net concentration of ethanol in the wort, g.L<sup>-1</sup>;

$C[TRS, f]$  = concentration of TRS in the fermented wort, g.L<sup>-1</sup>;

$C[TRS, i]$  = concentration of TRS in the wort, g.L<sup>-1</sup>;

$t$  = fermentation time, h.

Finally, 789g.L<sup>-1</sup> was the density value of the ethanol used and 0.511 was the sugar conversion factor proposed by the Gay-Lussac equation.

#### **4.9 Statistical analyses**

Analysis of variance (ANOVA) combined with Tukey's post hoc tests (statistical significance analysis with alpha value of 0.05) were performed. The statistical analyses and graphs were accomplished using the software excel, Minitab<sup>®</sup> 19 and python. It is noteworthy that except those mentioned, the experiments were performed in triplicate and the results were expressed as means followed by its standard deviation.



## 5 RESULTS AND DISCUSSION

### 5.1 Topic I: Feedstock physicochemical characterization

The physicochemical properties of corn hydrolysate and sugarcane syrup were investigated in this study. Glucose, fructose, sucrose, and maltose were the predominant fermentable sugar quantified in the feedstocks. To accurately determine the contribution of glucose and maltose to the Total Reducing Sugar (TRS) value, Equation 1 was employed, which involved dividing the amounts of sucrose and maltose by 0.95 prior to their addition to the TRS value<sup>80</sup>. The operation was required because during the yeast metabolism, the breakdown of maltose, and sucrose involves the hydrolysis of  $\alpha$ -glycosidic bonds, which releases a water molecule as part of the process<sup>81</sup>. Ultimately, the TRS value was calculated as the sum of these four sugars.

$$TRS = \sum Glucose + Fructose + \frac{(Maltose + Sucrose)}{0.95} \quad \text{Equation 1}$$

The carbohydrate content determined by ion chromatography is shown in Table 9.

Table 9 - Feedstock carbohydrate content determined by ion chromatography

<b>Analyses</b>	<b>Corn hydrolysate (g.L<sup>-1</sup>)</b>	<b>Sugarcane syrup (g.L<sup>-1</sup>)</b>
Glucose	369.09 ± 9.93	34.11 ± 1.84
Fructose	5.15 ± 0.59	46.16 ± 0.39
Sucrose	-	981.44 ± 35.94
Maltose	17.99 ± 0.72	-
TRS	393.18 ± 10.94	1,113.36 ± 37.59

Glucose emerged as the predominant fermentable sugar in the corn hydrolysate TRS composition, accounting for 93.9% of the total. This can be attributed to the enzymatic process utilized to produce corn hydrolysate, whereby the starch content within the endosperm is primarily hydrolyzed into glucose via the action of alpha-

amylase and amyloglucosidase enzymes. In contrast, maltose and fructose were present in relatively lower concentrations, representing approximately 4.8% and 1.3% of the corn hydrolysate TRS, respectively.

As expected, sucrose was the sugar with the highest contribution to sugarcane TRS composition, 92.8%, while fructose represented 4.1%. Unlike corn hydrolysate, the glucose in sugarcane was the sugar with the smaller contribution for the TRS composition, 3.1%. Finally, no maltose was detected in sugarcane syrup<sup>35,82</sup>.

As the sugarcane juice applied to prepare the sugarcane syrup was previously treated by heating, it concentrated the substances presented in the sugarcane juice. Therefore, sugarcane syrup exhibited 64.7% higher TRS concentration than corn hydrolysate. The aforementioned treatment is a plausible explanation for the higher values obtained for sugarcane syrup analyses in comparison to those of corn hydrolysate. This implication is evident in the results obtained from the total organic carbon analyzer described in Table 10.

Table 10 - Feedstock carbon and Nitrogen characterization by total organic carbon analyser

Analyses	Corn hydrolysate	Sugarcane syrup
Total organic Carbon -TOC (g. L <sup>-1</sup> )	151.86 T± 0.17	450.16 ± 12.34
Inorganic Carbon - IC (g. L <sup>-1</sup> )	0.15 ± 0.01	0.16 ± 0.02
Total Carbon - TC (g. L <sup>-1</sup> )	152.01 ± 45.7	450.32 ± 12.4
Total Nitrogen - TN (g. L <sup>-1</sup> )	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	800.98 ± 48.91

Based on the research conducted by Manikandan and Viruthagiri<sup>83</sup>, the optimal C:N ratio for ethanol production using *Saccharomyces cerevisiae* in tapioca starch is 35.2. Taking this to account, it is possible to infer that corn hydrolysate and sugarcane syrup were rich in carbon, but poor in Nitrogen for ethanol production. However, it is important to note that this ratio may vary depending on the specific fermentation

process being used. In many cases, the C:N ratio required for tapioca starch may not be suitable, mainly in process where the yeast cells are often recycled, and the fermenters contain high cell density. For instance, in the Melle-Boinot process, a high Nitrogen concentration can increase yeast biomass production but decrease fermentation yield.

Furthermore, the total organic carbon results were thousand times higher than the inorganic carbon, mainly because the feedstocks are mostly composed of sugars, and sugars are included in the organic carbon composts.

Similarly, the Nitrogen content, macronutrients, and micronutrients, such as Potassium and Manganese, have the potential to affect yeast metabolism<sup>84</sup>. Therefore, the levels of Potassium, Phosphorus, Magnesium, Sodium, Calcium, Zinc, Iron, Copper, Cobalt, and Manganese present in both raw materials were evaluated using ICP OES. It is noteworthy to state that fluctuations in sugarcane syrup and corn hydrolysate composition may occur due to aspects such as variety of soil, seed and climate<sup>34</sup>.

In this context, it is crucial to consider the origin of sugarcane juice, as it can significantly impact its composition. Industrial sources of sugarcane juice typically exhibit higher levels of Calcium (Ca) and Magnesium (Mg) than those obtained from street vendors. Distilleries frequently employ calcitic or dolomitic lime to adjust the pH level and clarify the juice, resulting in a greater concentration of Ca and Mg in the clarified sugarcane juice compared to that supplied by street vendors. Additionally, several distilleries blend juice and molasses, which further augments the Ca and Mg content in the wort.

The total concentration of each nutrient assessed is shown in Table 11. As the method applied quantifies the nutrient total concentration, some nutrients may be present in the feedstock in not assimilable forms for yeast metabolism<sup>34</sup>.

Table 11 - Feedstock physicochemical characterization by inductively coupled plasma optical emission spectrometry - (ICP OES)

<b>Nutrient analyzed</b>	<b>Sugarcane syrup (mg.L<sup>-1</sup>)</b>	<b>Corn hydrolysate (mg.L<sup>-1</sup>)</b>	<b>Recommended level (mg.L<sup>-1</sup>)<sup>74,75</sup></b>
Potassium	1,633.9 ± 65.1	702.5 ± 22.6	750.0
Phosphorus	485.7 ± 18.2	564.3 ± 11.7	311.0
Magnesium	316.8 ± 31.6	144.3 ± 7.1	135.0
Sodium	277.4 ± 33.9	67.8 ± 7.9	200.0
Calcium	146.1 ± 5.5	30.5 ± 3.0	120.0
Zinc	11.4 ± 1.5	1.5 ± 0.0	5.3
Iron	11.4 ± 1.5	2.2 ± 0.8	0.2
Copper	12.3 ± 1.5	2.7 ± 0.8	7
Cobalt	9.6 ± 1.5	2.3 ± 0.3	10
Manganese	5.3 ± 0.0	2.3 ± 0.6	21.5

To compare the nutrient levels above and below the recommended range for alcoholic fermentations in the corn hydrolysate and in the sugarcane syrup, a comparison matrix was created and evidenced in Figure 15. It is important to highlight that the matrix was created by comparing the nutrient concentrations obtained from the feedstock characterization with the recommended concentrations described by Santos and the company fermentec<sup>18,19</sup>.

As observed in Table 11 and Figure 15 for both feedstocks, Phosphorus, Magnesium, and Iron exceed the recommended levels for ethanol production, whereas Manganese and Cobalt were under the recommended range proposed in the literature<sup>74,75</sup>.

<b>Above</b>	P, Fe <sup>++</sup> , and Mg <sup>++</sup>	K <sup>+</sup> , Na <sup>+</sup> , Ca <sup>++</sup> , Zn <sup>++</sup> , Fe <sup>++</sup> , Cu <sup>++</sup> , P, and Mg <sup>++</sup>
<b>Below</b>	NH <sub>4</sub> <sup>+</sup> , K <sup>+</sup> , Na <sup>+</sup> , Ca <sup>++</sup> , Zn <sup>++</sup> , Fe <sup>++</sup> , Cu <sup>++</sup> , Co <sup>++</sup> , and Mn <sup>++</sup>	NH <sub>4</sub> <sup>+</sup> , Co <sup>++</sup> , and Mn <sup>++</sup>
	<b>Corn Hydrolysate</b>	<b>Sugarcane Syrup</b>

Figure 15 - Comparison matrix of the nutrient concentration range in both raw material

Lastly, the pH and density were evaluated for corn hydrolysate and sugarcane syrup. As shown in Table 12, both feedstocks present a similar pH and it was slightly above the optimal pH for *S. cerevisiae* fermentation, which is settled between 4.0-5.0<sup>12</sup>.

Table 12 - Feedstock physicochemical analyses

<b>Analyses</b>	<b>Corn hydrolysate</b>	<b>Sugarcane syrup</b>
pH	5.80 ± 0.04	5.70 ± 0.02
Density (kg.m <sup>-3</sup> )	1,099 ± 0.01	1,380 ± 0.02

## 5.2 Topic II: Assessment of nutrients' supplementation impacts on alcoholic fermentation

In this section, the focus was on discussing the results of fermentation experiments conducted in two different setups: microplate for ELISA, and conical tubes. The aim was to assess the effects of nutrient supplementation on the fermentation of corn and sugarcane mixed wort without any decontamination methods applied.

The experimental design involved twelve different conditions, eleven of which were supplemented with a different nutrient, while one served as the control without any supplementation. To ensure a fair comparison, all the experimental conditions were kept constant across the tests in both setups. This included maintaining the same inoculum concentration, temperature, agitation, volume, and fermentation time.

The experimental conditions tested in both setups (microplate for ELISA, and fermentation in conical Tubes) from section 5.2 are shown in Figure 16.

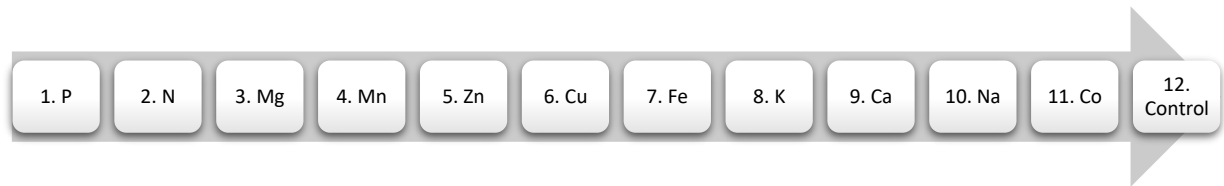


Figure 16 - Experimental conditions tested in both setups from section 5.2

### 5.2.1 Fermentation in microplate for ELISA

This study involved conducting alcoholic fermentations in microplates for ELISA to evaluate yeast growth under twelve different conditions, both with and without supplementation. The fermentations were monitored for 18.3h, when the yeast reached the stationary phase for all conditions. Despite the addition of 10 $\mu$ L of the Ethanol Red inoculum to each well, variability was observed in the initial absorbance levels among the conditions under evaluation. However, all conditions ultimately yielded a final absorbance of up to 0.43. By establishing a correlation between dry mass and absorbance, the study determined that approximately 15mg of Ethanol Red biomass was produced based on dry mass.

A comparison between the Ethanol Red's growth profile in mixed wort without supplementation and supplemented with Nitrogen, Potassium, Phosphorus, Magnesium, Sodium, Calcium, Zinc, Iron, Copper, Cobalt, and Manganese is shown in Figure 17. The results indicate that Ethanol Red was able to growth under all tested conditions.

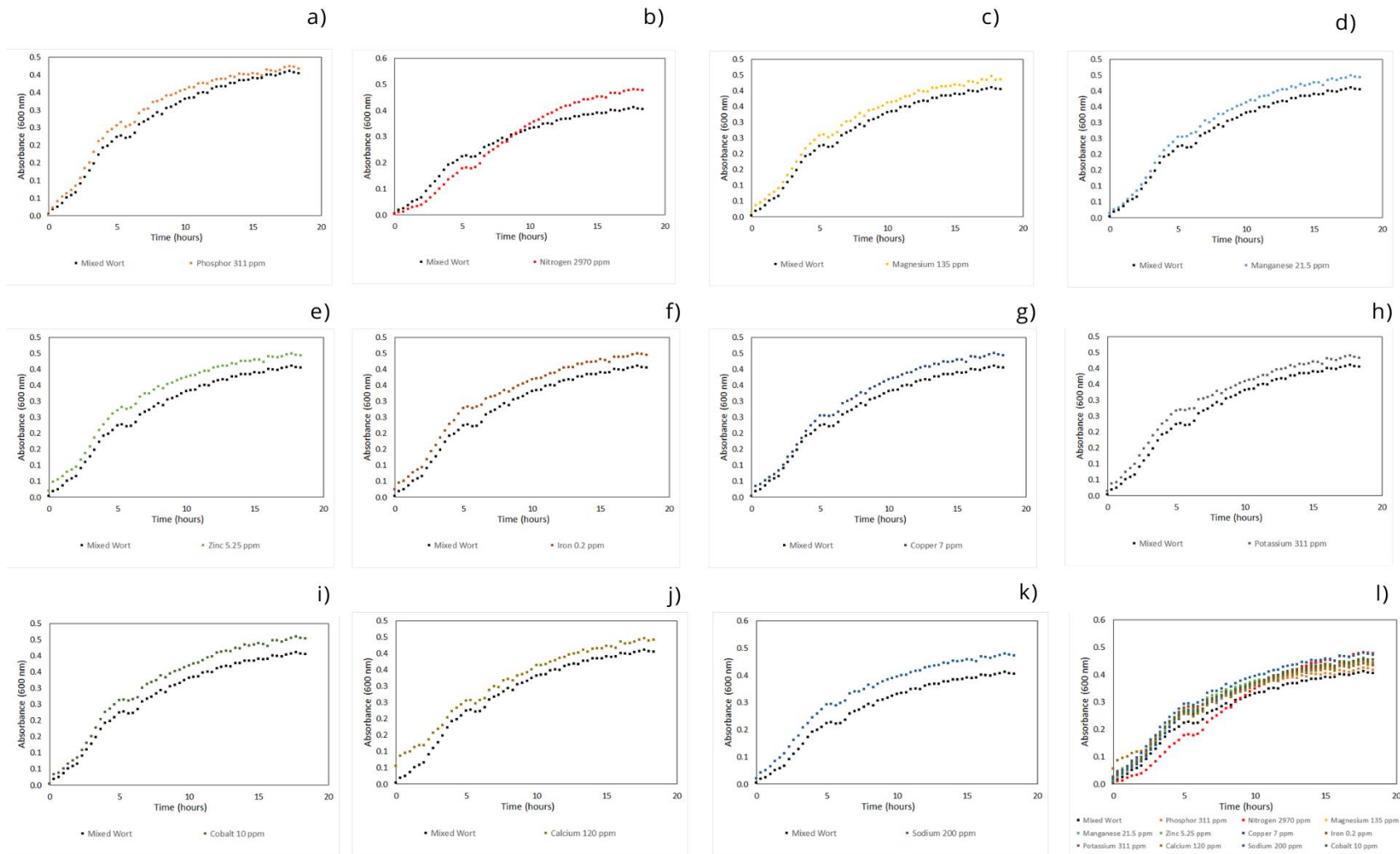


Figure 17 - Analysis comparing the yeast growth profile in mixed wort supplemented with a) Phosphorus, b) Nitrogen, c) Magnesium, d) Manganese, e) Zinc, f) Iron g) Copper, h) Potassium, i) Cobalt, j) Calcium and k) Sodium. l) Yeast growth profile evaluated for all twelve conditions

The increase in yeast specific growth rate represents advances for the ethanol industrial process, owing to its potential to curtail the fermentation duration and escalate the ethanol productivity<sup>84</sup>. Moreover, improvements in yeast-specific growth rate help the yeast to multiply faster, dominate, and persist in the process. To numerically evaluate the nutrient impacts on yeast metabolism, the specific growth rate of Ethanol Red was calculated for each condition.

As can be seen in Figure 18, Nitrogen, Cobalt, Sodium, Phosphorus, Magnesium, Potassium, Zinc, Copper, and Manganese revealed an increase in the yeast specific growth rate. This result suggests that the nutrients mentioned above might contribute to increasing the alcoholic fermentations' yield. Moreover, Nitrogen was the nutrient with the highest positive impact in the yeast growth rate, up to 49% in relation to mixed wort without supplementation (control).

In addition to the increase in the yeast's specific growth rate, the nutrients mentioned above can also promote the growth of microbial contaminants, especially in fermentations carried out without sterilization.

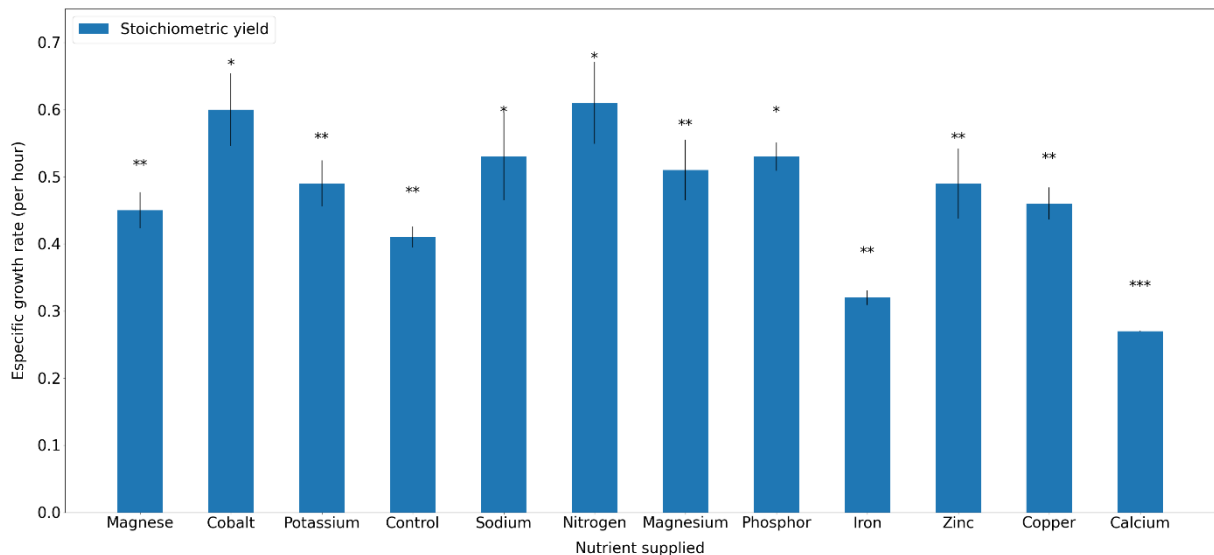


Figure 18 - Assessment of nutrient supplementation in the Ethanol Red's specific growth rate. Asterisks denote whether the averages of specific growth rate values are statistically higher (\*), similar (\*\*), or lower (\*\*\*) than the Control sample

Based on Figure 18, the yeast's specific growth rate decreased by over 22% upon exposure to Calcium. Calcium is recognized for its ability to enhance yeast's ethanol stress tolerance during alcoholic fermentations, and the element is also



involved in activities related to the membrane's function and structure<sup>10,75,84</sup>. Despite ongoing discussions regarding Calcium's optimal level, excessive amounts of this element can disrupt the amino acids and Magnesium uptake, resulting in a blockade of cell activities that require  $Mg^{2+}$  to enter the cell<sup>84</sup>.

As described in Table 5, Calcium concentration of around  $120mg.L^{-1}$  is necessary for achieving adequate alcoholic fermentation. However, the optimal Calcium levels required for yeast growth remains a topic of discussion, with some studies suggesting a concentration of around  $180mg.L^{-1}$ .

Upon examination of Table 11, it was found that sugarcane syrup contains a higher Calcium concentration than the recommended level for alcoholic fermentation (which is  $146mg.L^{-1}$ ), but still falls within the optimal range for yeast growth ( $180mg.L^{-1}$ ). Additionally, it is shown in Figure 18 that yeast's specific growth rate was decreased with Calcium supplementation. Based on these findings, it is possible to conclude that sugarcane syrup is likely to be a suitable feedstock for alcoholic fermentation and yeast growth without the need for additional Calcium supplementation to support the processes.

Furthermore, it is evidenced in Figure 18 a decrease in yeast specific growth rate due to Iron supplementation. When comparing with the feedstock characterization from Table 5, it becomes evident that Iron exceeded the recommended level for adequate alcoholic fermentation only in the corn hydrolysate. This highlights the fact that corn hydrolysate may be also a suitable feedstock for alcoholic fermentation without any further Iron supplementation.

### **5.2.2 Fermentation in conical tubes**

Fermentation in conical tubes was carried out until tubes' mass reached less than 0.02g which lasted for 52h. According to the theoretical conversion proposed by Gay-Lussac, only 51.1% of the fermentable sugars in the wort can be converted to ethanol by the yeast. Moreover, as part of the sugar is deviated to other cell activities such as the glycerol, biomass and organic acids production, the theoretical conversion is hardly achieved. The yeast metabolism during alcoholic fermentation is summarized shown in Figure 19.

Overall, this investigation provides valuable insights into the complex processes involved in fermentation and underscores the importance of understanding the interplay between various cellular functions in achieving optimal conversion rates. The metabolic pathways of yeast during alcoholic fermentation are summarized in Figure 19.

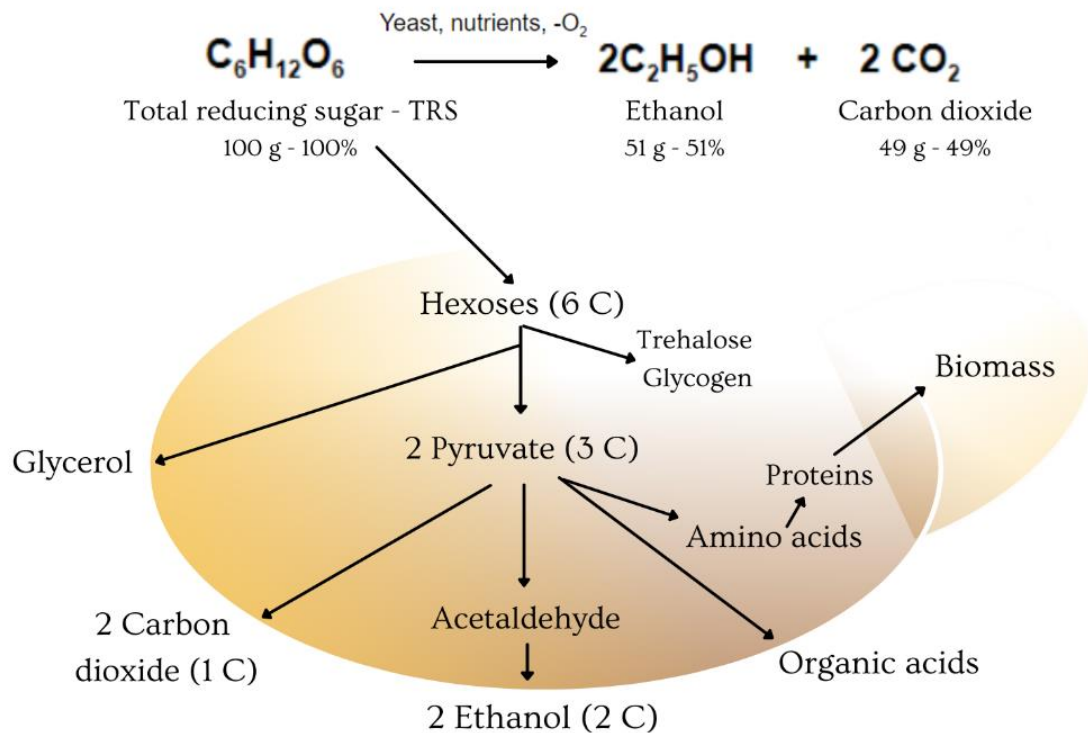


Figure 19 – Summarized representation of yeast's metabolic pathways during alcoholic fermentation

Stuck and sluggish fermentation are commonly associated with deficiencies in Nitrogen, Magnesium, and Zinc or an excess of Calcium<sup>10</sup>. However, the present investigation demonstrated that only mixed wort supplemented with Nitrogen achieved a conversion rate exceeding 99% of the initial TRS, while the other conditions exhibited more than  $32g.L^{-1}$  of residual TRS, equivalent to nearly 26% of the initial TRS. This highlights the significance of Nitrogen supplementation in corn and sugarcane mixed wort fermentations with low inoculum concentrations ( $3-4\%w.w^{-1}$ ) and without recycling. Nevertheless, it should be noted that even with Nitrogen supplementation, a portion of the TRS consumed may have been redirected towards biomass, glycerol, storage carbohydrates, and fermentation by-products<sup>10</sup>. Microbial contamination and

parallel reactions, such as Maillard reactions, are also known to contribute to sugar consumption<sup>10</sup>.

In the Nitrogen-supplemented condition, fermentation started at  $123\text{g.L}^{-1} \pm 2$  of TRS, resulting in the production of  $53\text{g.L}^{-1} \pm 1$  of ethanol. Further details about the initial and residual TRS, as well as the production of ethanol and glycerol under each evaluated condition, are provided in Figure 20. Notably, all comparisons were made with reference to the control condition, which lacked any form of supplementation.

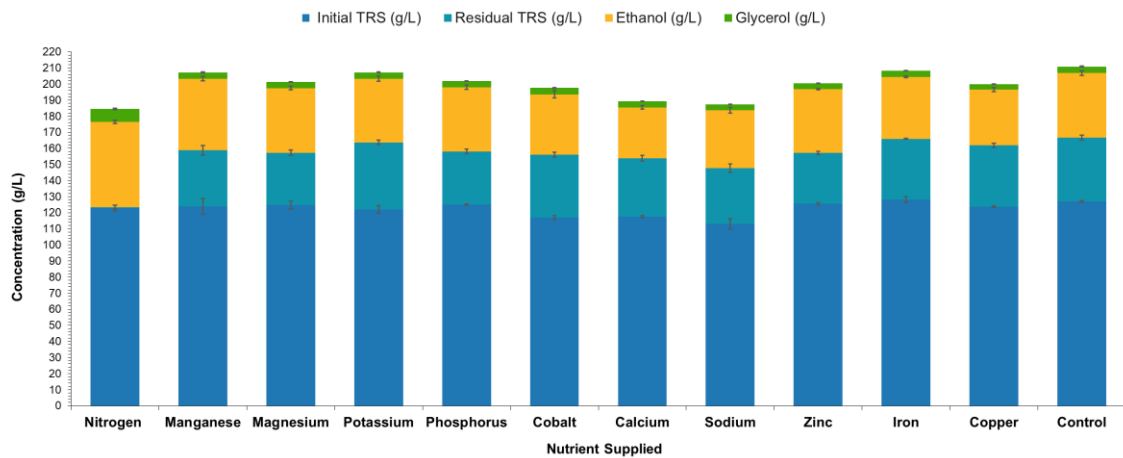


Figure 20 - Assessment of nutrient supplementation in the ethanol, glycerol, and residual TRS obtained in the fermented wort versus the initial TRS

The assimilable forms of Nitrogen in the medium are absorbed by the yeast to produce essential biomolecules, including but not limited to peptides, proteins, polyamides, nucleic acids, and vitamins, all of which are crucial for yeast multiplication<sup>86,87</sup>. Consequently, the presence of Nitrogen in the medium accelerates yeast catabolism, thereby increasing ATP production to support cell maintenance and growth. In addition, this upsurge in metabolic activity is accompanied by increased consumption of hexoses, ultimately resulting in greater excretion of ethanol and carbon dioxide into the medium.

In anaerobic conditions, glycerol functions not only as a cellular growth and osmotic stress-associated factor<sup>34</sup>, but also as an electron acceptor for maintaining NADH redox balance<sup>88,89</sup>. Approximately 10% of the total reducing sugars (TRS) supplied are converted to glycerol during alcoholic fermentation. In this experiment, the introduction of high concentrations of ammonium sulfate ( $2.97\text{g.L}^{-1}$ ) created osmotic stress, leading to an elevation in glycerol production by the yeast. The glycerol

concentration in the fermented wort containing Nitrogen ( $8\text{g.L}^{-1} \pm 0.4$ ) was twice as high as that observed in all other conditions ( $4\text{g.L}^{-1} \pm 1.2$ ). However, despite the increased glycerol production, the glycerol content remained below 10% of the initial TRS in the mixed wort for all conditions. It is possible that, as other nutrient factors became limiting to yeast growth, salt stress may have contributed more to glycerol production than yeast growth.

The calculation of ethanol yield was performed using two distinct methods: stoichiometry and technological yield, as presented in Table 8. According to the results of statistical analyses, Calcium was found to be the only nutrient that diminished both the yeast-specific growth rate and the fermentative parameters. As previously discussed, Calcium has been shown to have detrimental effects on yeast metabolism. In addition to Calcium, Iron, Zinc, and Copper were found to have an adverse effect on at least one of the calculated fermentative parameters.

The presence of excess Copper and Iron has been observed to induce cell death<sup>48,84</sup>, although the effect of their presence can vary depending on the specific strain of yeast<sup>84</sup>. Iron, which functions as a cofactor in yeast respiratory activity and growth<sup>75</sup> due to its role as an enzyme catalytic center, can also have negative consequences when present in excess, leading to a reduction in enzymatic activities, such as those of pyruvate and succinate dehydrogenases, and ultimately resulting in cell death<sup>84</sup>. Additionally, excessive accumulation of Zinc within the cell can result in toxicity, attributable to metabolic pathway suppression, competition with other metals for enzyme active sites, and improper binding with intracellular ligands<sup>85, 84</sup>.

Despite the observed improvement in the yeast's specific growth rate upon addition of Cobalt and Sodium, there was no corresponding increase in ethanol yield. This finding suggests that the presence of these elements may have influenced the allocation of supplied TRS towards biomass production, rather than ethanol production.

In the context of alcoholic fermentation, the addition of nutrients to the fermentation medium can play a crucial role in determining the efficiency and quality of the process. To investigate this, the impacts of nutrient supplementation on three response variables, namely productivity, stoichiometric yield, and technological yield,

were analyzed. The results regarding these three variables are presented below for all experimental conditions, as evidenced in Figure 21.

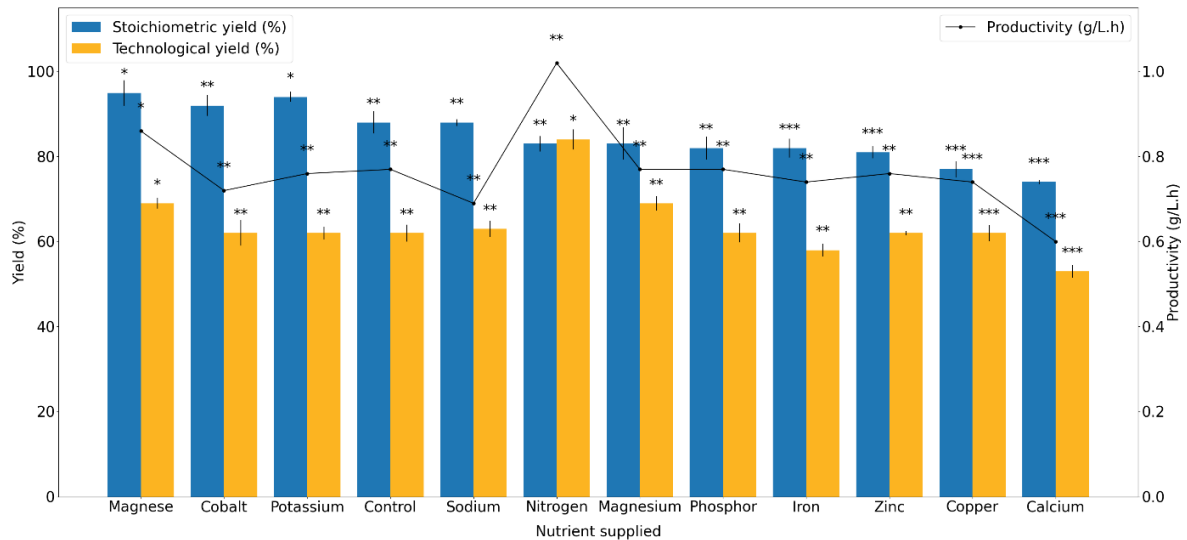


Figure 21 – Assessment of nutrient supplementation in the fermentative parameters. For each response variable, productivity, stoichiometric and technological yield, asterisks indicate if averages are statistically higher (\*), similar (\*\*), or lower (\*\*\*) than the Control sample

About the technological yield, Nitrogen, Manganese, and Potassium supplementation returned increases on it. As the carbon/Nitrogen ratio (Table 10) for both feedstocks were above the described level for ethanol production (35.2 C/N ratio)<sup>83</sup>, it was expected that Nitrogen could increase the ethanol technological yield.

The Nitrogen addition resulted in the highest yeast-specific growth rate (49%), productivity (32%), and technological ethanol yield (35%). Despite this, Manganese and Potassium had the most significant positive impact on the stoichiometry yield. This could be attributed to the fact that unlike the technological yield, the stoichiometry yield considers the TRS balance at the beginning and end of fermentation. Both conditions resulted in a stoichiometry yield higher than current values observed in the bioethanol industry (90-92%)<sup>91</sup>, with a yield range between 74% to 92%.

Regarding the Manganese, and Potassium roles in the cell, Manganese acts as a cofactor for enzymatic activities, stimulates yeast growth, and enhances fermentation<sup>84</sup>. Similarly, Potassium is involved in both yeast anabolism and catabolism<sup>92</sup>, and the element plays a crucial role in improving yeast tolerance to

ethanol stress, acts as a cofactor, and facilitates the uptake of nutrients such as phosphates<sup>84,92</sup>.

Bioethanol is considered a primary metabolite, indicating a direct link between ethanol production and yeast growth kinetics<sup>93</sup>. This correlation is illustrated in Figure 17, which shows a similarity between the ethanol production curve and the growth kinetics of Ethanol Red yeast<sup>93</sup>.

Another implication is related to the stress upon the yeast during alcoholic fermentations, particularly when ethanol content is produced in concentrations exceeding 10% w.v<sup>-1</sup><sup>94</sup>. Ethanol's toxic effects can significantly reduce the metabolic activity of yeast, resulting in impaired cell viability, growth, and membrane structure, and function.

The fermentation process with Nitrogen supplementation resulted in the highest ethanol content of 7%  $\pm$  0.1. However, the concentration was below the recommended levels to avoid detrimental effects on the yeast physiology caused by excess ethanol. The alcohol content for all experimental conditions was calculated based on v.v<sup>-1</sup> and is presented in Figure 22.

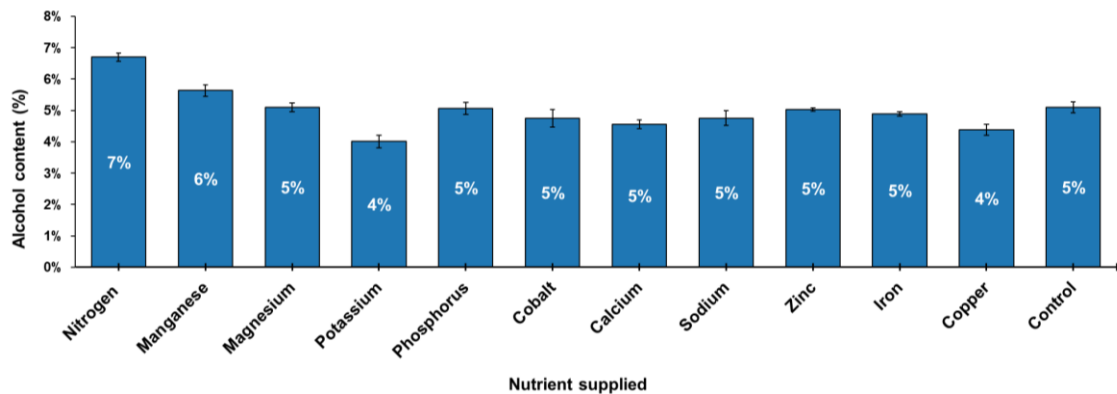


Figure 22 - Ethanol content presented in the fermented mixed wort with and without supplementation

Lactic acid production in fermented wort is mainly attributed to the metabolic activity of microbial contaminants, such as *Bacillus* and *Lactobacillus* bacteria<sup>14,87</sup>. However, yeasts also contribute in small amounts to its synthesis<sup>90</sup>. The average concentration of lactic acid detected in the fermented wort was 0.3g.L<sup>-1</sup>  $\pm$  0.1, except for the Phosphorus condition, which showed a concentration of 0.6g.L<sup>-1</sup>  $\pm$  0.1. This

finding suggests that the microbial activities during fermentation did not reach levels that would cause stress to the yeast, which is typically observed at concentrations between 0.2-0.8% w.v<sup>-1</sup>.

The experimental findings demonstrate that Nitrogen supplementation during the fermentation process resulted in the highest acetic acid concentration, measuring at 0.8g.L<sup>-1</sup> ± 0.1 (0.0008% w.v<sup>-1</sup>). In contrast, all other conditions yielded values either equivalent to or below 0.2g.L<sup>-1</sup> ± 0.2 (0.0002% w.v<sup>-1</sup>). Of particular note, the levels of acetic acid observed in the study remained consistently below the known concentrations that can induce yeast stress, typically falling within the range of 0.05-0.1% w.v<sup>-1</sup><sup>95</sup>. It is worth noting that while microbial contamination is the primary source of lactic acid and acetic acid, the latter can also be endogenously synthesized by *Saccharomyces Cerevisiae*.

The succinic acid concentrations measured in the fermented wort (1.4g.L<sup>-1</sup> ± 0.2) were found to be lower than the estimated levels for *Saccharomyces Cerevisiae* alcoholic fermentation, which can reach up to 1.7g.L<sup>-1</sup><sup>96,97</sup>. Succinic acid is often used as a microbial contamination indicator, as it is produced by yeast to suppress bacterial activity<sup>46, 98</sup>. Additionally, it is worth noting that the application of sugarcane treatment via heating may have contributed to reducing the microbial population in the feedstock. The organic acids acetic, lactic, and succinic were evaluated in this study and are presented in Figure 23.

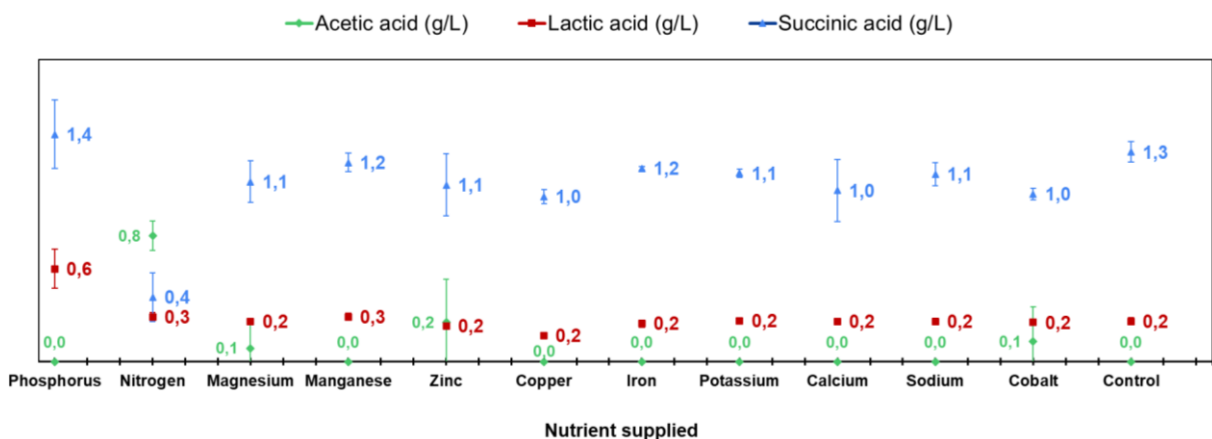


Figure 23 - Organic acids concentrations in the fermented mixed wort with and without supplementation

Mannitol is a microbial contamination indicator commonly found in fermented wort, with its production primarily attributed to bacterial contamination<sup>14</sup>. However, in this study, the measured mannitol concentrations were found to be below the calibration range of the ion chromatograph. This suggests that microbial activity did not have a significant impact on ethanol production, thus indicating the absence of substantial bacterial contamination in the fermented mixed wort<sup>99</sup>.

### **5.3 Topic III: Assessment of wort decontamination**

This section covered four subsections, namely the evaluation of the impact of electron beam on carbohydrate content, evaluation of electron beam and antibiotic impacts on wort decontamination, fermentation in microplate for ELISA, and fermentation in conical tubes.

The tests were performed using corn and sugarcane mixed wort, without any supplementation, and were decontaminated through the application of Kamoran HJ antibiotic or electron beam at 10kGy, 15kGy, and 20kGy. The results were compared with the control, which represents the condition without any wort decontamination or supplementation.

To ensure a fair comparison, the experimental conditions were kept the same during each setup in all the subsections, including inoculum concentration, temperature, agitation, volume, and fermentation time. The experimental design for all the tests conducted in the four subsections is illustrated in Figure 24.



#### 5.3.1 Assessment of electron beam treatment on carbohydrate content

- **Conditions (4):** Control, mixed wort decontaminated by e-beam at 10kGy, 15kGy, and 20kGy.
- **Objective:** Discuss the results from carbohydrate content before and after mixed wort treatment with e- beam.
- **Parameters:** TRS concentration (glucose, fructose, sucrose, and maltose).

#### 5.3.2 Evaluation of electron beam and antibiotic treatment on wort decontamination

- **Conditions (5):** Control, mixed wort decontaminated by Kamoran HJ, e-beam at 10kGy, 15kGy, and 20kGy.
- **Objective:** Discuss the efficiency of antibiotic, and e-beam to reduce microbial contamination in the mixed wort.
- **Parameters:** Concentration of microbial contamination, in terms of CFU.mL<sup>-1</sup>.

#### 5.3.3 Fermentation in microplate for ELISA

- **Conditions (5):** Control, mixed wort decontaminated by Kamoran HJ, e-beam at 10kGy, 15kGy, and 20kGy.
- **Objective:** Discuss the effects of electron beam and antibiotics on Ethanol Red growth.
- **Parameters:** Ethanol Red's growth profile (absorbance values), and specific growth rate.

#### 5.3.4 Fermentation in conical tubes

- **Conditions (5):** Control, mixed wort decontaminated by Kamoran HJ, e-beam at 10kGy, 15kGy, and 20kGy.
- **Objective:** Discuss the results from the parameters evaluated during ethanol production and yeast growth.
- **Parameters:** Productivity, stoichiometric, and technological yield, alcohol content, cell viability, bacterial contamination, TRS, glycerol, organic acids, mannitol, and ethanol concentration.

Figure 24 - Experimental design for the studies in the section 5.3

### 5.3.1 Assessment of electron beam treatment on carbohydrate content

Ion chromatography technique was employed to evaluate the impact of electron beam treatment on the carbohydrate content of mixed wort, with glucose, fructose, sucrose, and maltose being the targeted carbohydrates. Thereafter, the TRS was calculated for each condition. The results obtained for the samples irradiated by electron beam at 10kGy, 15kGy, and 20kGy were compared with the values obtained for the untreated mixed wort (0kGy).

Maltose and fructose concentrations remained with the same concentrations before and after electron beam treatment. Glucose, sucrose, and consequently, the TRS concentrations obtained for the irradiated samples were slighted above the untreated sample. However, considering the margin of error, it is possible to infer that all samples presented the same carbohydrate content. The carbohydrate content for all conditions evaluated is shown in Table 13.

Table 13 - Evaluation of electron beam impacts on carbohydrate content

Variable	0kGy	10kGy	15kGy	20kGy
Glucose (g.L <sup>-1</sup> )	139 ± 6	140 ± 5	144 ± 5	143 ± 4
Fructose (g.L <sup>-1</sup> )	3 ± 1	3 ± 0	3 ± 0	3 ± 0
Sucrose (g.L <sup>-1</sup> )	9 ± 1	10 ± 1	10 ± 1	11 ± 1
Maltose (g.L <sup>-1</sup> )	1 ± 0	1 ± 0	1 ± 0	1 ± 0
TRS (g.L <sup>-1</sup> )*	152 ± 6	156 ± 6	159 ± 5	158 ± 4

Additionally, experiments involving sugarcane syrup irradiated up to 80kGy have shown that electron beam treatment does not interfere with the glucose, sucrose, and fructose content<sup>14</sup>. However, no studies have been found regarding the impact of electron beam treatment on carbohydrate content in mixed wort containing both corn and sugarcane.

### 5.3.2 Evaluation of electron beam and antibiotic treatment on wort decontamination

The efficiency of electron beam and Kamoran HJ treatments to reduce the microbial contamination in mixed wort were evaluated in Petri dishes containing YPD broth. It was noticed that the untreated mixed wort presented microbial contamination around 10<sup>4</sup>CFU.mL<sup>-1</sup>. Both Kamoran HJ treatment and electron beam irradiation at 10kGy reduced the microbial contamination to approximately 10<sup>2</sup>CFU.mL<sup>-1</sup>. Further, electron beam treatment at 15kGy kept the microbial contamination under 10<sup>1</sup>CFU.mL<sup>-1</sup>, while 20kGy led to the mixed wort sterilization.

According to the supplier, Kamoran HJ can reduce up to 80% of gram-positive bacteria, including the lactobacillus genus, which is responsible for a significant proportion of microbial contamination during alcoholic fermentations. As the analyses carried out were based on the reduction of total microbial contamination, measured in CFU.L<sup>-1</sup>, it was observed that Kamoran HJ reduced approximately 97.93% of the total microbial load. Moreover, the results showed that e-beam treatment at 10kGy, 15kGy, and 20kGy was highly effective, with reductions in total microbial contamination of approximately 99.21%, 99.99%, and 100%, respectively.

The total microbial contamination in CFU.mL<sup>-1</sup> and in log (CFU.mL<sup>-1</sup>) for mixed wort with no treatment, electron beam and antibiotic treatment are shown in Figure 25.

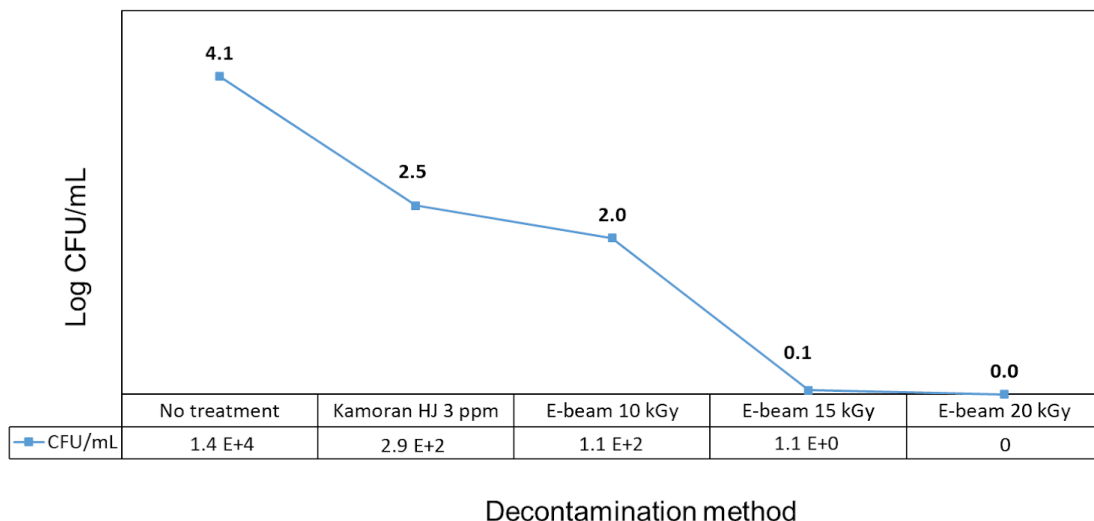


Figure 25 - Total microbial contamination in the mixed wort without treatment and treated by electron beam, and antibiotic

The electron beam treatment exhibited a continuous reduction of the total microbial contaminants (CFU.mL<sup>-1</sup>) in the mixed wort, indicating a decrease in various fungi and bacterial strains. According to Pinto<sup>100</sup> and Vasconcelos<sup>101</sup>, commercial sterilization must achieve a reduction of 99.99% in total microbial contamination, which typically requires ionizing irradiation doses ranging from 10 to 45kGy to reduce approximately 10<sup>4</sup> CFU.mL<sup>-1</sup> 15. As illustrated in Figure 25, both electron beam irradiation at 15kGy and 20kGy achieved the commercial sterilization standard. However, only the 20kGy dose led to the mixed wort sterilization.

In studies carried out with sugarcane wort containing external microbial contamination ( $10^7\text{CFU.mL}^{-1}$ ), Silva<sup>15</sup> detected 80kGy as the electron beam dose to sterilise the wort, while a dose of 40kGy was required for commercial sterilization. Lastly, 20kGy was able to reduce  $7.9\times 10^3\text{CFU.mL}^{-1}$  in the sugarcane wort. The difference in the electron beam doses required to achieve sterilization between the present study and Silva<sup>15</sup> could be attributed to the initial microbial contamination in each wort. The total microbial contamination in the sugarcane wort was nearly double ( $10^7\text{CFU.mL}^{-1}$ ) that found in the mixed wort ( $10^4\text{CFU.mL}^{-1}$ ). This could be attributed to the fact that Silva<sup>15</sup> study introduced external contaminants from soil into the sugarcane wort, while the present study was performed with the natural microbial contamination present in the mixed wort.

### **5.3.3 Fermentation in microplate for ELISA**

The alcoholic fermentations in microplates for ELISA were conducted to evaluate the effects of electron beam and antibiotics on Ethanol Red growth. Regardless of the decontamination method applied, Ethanol Red required the same time to adapt and express the genes required for growth in the mixed wort. This means that the lag phase lasted about two hours for all evaluated conditions.

The log phase was observed to occur between two to six hours. During the exponential phase, the mixed wort treated by an electron beam and Kamoran HJ exhibited a steeper slope than the untreated condition. The deceleration phase extended to twelve hours, followed by the stationary phase that persisted until the end of cultivation, approximately 18 hours. A comparison between the Ethanol Red's growth profile in untreated mixed wort and treated by electron beam and Kamoran HJ is shown in Figure 26.

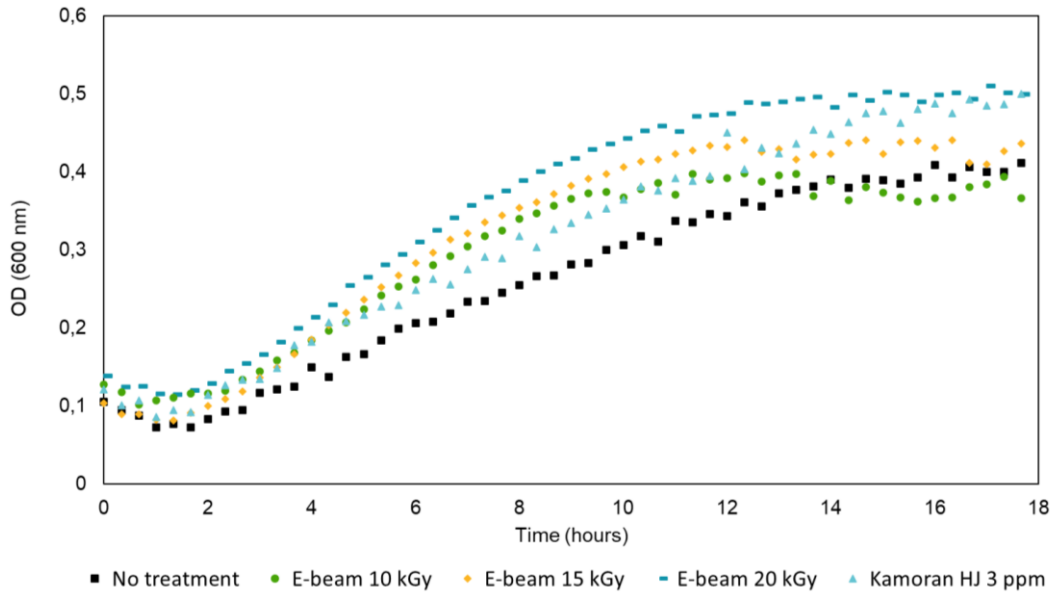


Figure 26 - Comparison between Ethanol Red growth profile in mixed wort without treatment and treated by Kamoran HJ, electron beam at 10kGy, 15kGy and 20kGy

Notably, only the mixed wort treated with e-beam at 20kGy and 15kGy significantly impacted the yeast-specific growth rate, showing a 54.1% and 45.8% increase, respectively, based on a statistical analysis with 5% of significance level. Mixed wort treated with e-beam at 10kGy and Kamoran HJ showed higher yeast-specific growth rates than the untreated condition, although these values fell within the same range than the control when considering the statistical analysis. The yeast specific growth rate calculated for all decontamination methods is shown in Figure 27.

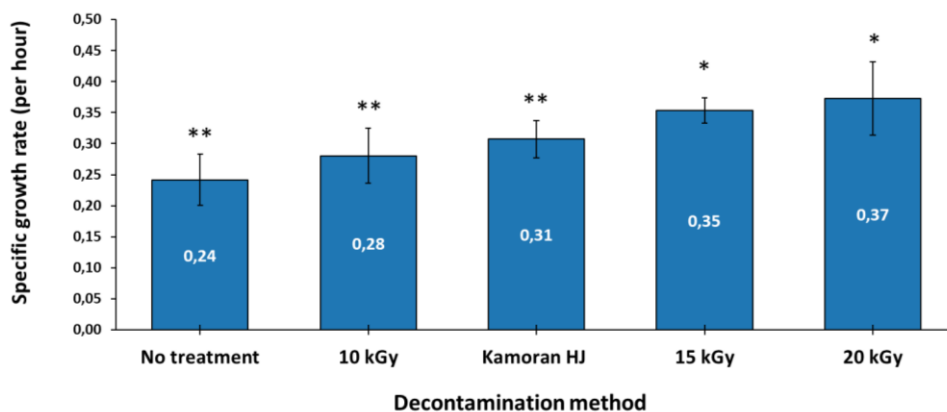


Figure 27 – Effects of mixed wort decontamination in the Ethanol Red's specific growth rate. For specific growth rate values, asterisks indicate if averages are statistically higher (\*), similar (\*\*), or lower (\*\*\*) than the Control sample

As seen in Figure 26 and 27, the electron beam action in the mixed wort positively impacted the Ethanol Red specific growth rate. The discussed hypothesis is

that like other techniques such as ultrasound, the electron beam can break down macromolecules in the wort and release the nutrients required for yeast growth<sup>102,103</sup>. Additionally, electron beam irradiation can enhance yeast growth by eliminating microbial contaminants present in the mixed wort, such as bacteria, fungi, protozoa, and viruses<sup>20</sup>. Furthermore, it can damage the genetic material of cells and affect vital functions like reproduction<sup>20</sup>. As a result, more nutrients and sugars are available in the medium for yeast growth and yeast competition for the substrate is reduced<sup>20</sup>.

In the context of fermentation processes, the use of various treatments on mixed wort can have a significant impact on the yeast's growth and viability. The results showed that the mixed wort treated with electron beam at 15kGy and 20kGy returned higher yeast specific growth rates than the condition with Kamoran HJ. This finding is likely because the action of Kamoran HJ is primarily aimed at eliminating gram-positive bacteria, while the electron beam treatment affects the entire microbial community in the wort<sup>104</sup>.

Furthermore, e-beam interaction with the wort composition also seems to improve the nutrients availability in the medium for yeast growth. This suggests that the electron beam treatment may have a broader impact on the composition and quality of the wort, beyond its effect on microbial populations.

In conclusion, the results of this study suggest that the use of electron beam irradiation as a treatment for mixed wort may be a more effective method for promoting yeast growth and viability than the use of Kamoran HJ. These findings underscore the importance of considering the broader effects of treatment methods on the composition and quality of fermentation substrates, beyond their direct impact on microbial populations. Further research is needed to fully understand the mechanisms underlying these effects and to optimize treatment methods for various types of fermentation substrates. Lastly, data on ethanol production in these conditions are presented in the following subsection.

### **5.3.1 Fermentation in conical tubes**

The fermentation of mixed wort in conical tubes was monitored in this study for 43 hours by measuring CO<sub>2</sub> shedding. The results revealed that after fermentation,

around 8% of the initial total reducing sugars (TRS) remained in the fermented mixed wort, with similar residual TRS levels observed across all tested conditions ( $10\text{g.L}^{-1} \pm 1$ ). These findings suggest that while decontamination methods are crucial for maintaining a clean fermentation environment, they may not necessarily prevent stuck fermentation. A comparison of the findings in Figure 28, where no additional supplementation was provided, with those in Figure 20 (supplementation study), reinforce the conclusion that nutrient supplementation, specifically Nitrogen, plays a vital role in promoting complete fermentation.

All information about the initial and residual TRS, ethanol and glycerol measured in the mixed wort before and after fermentation is shown in Figure 28.

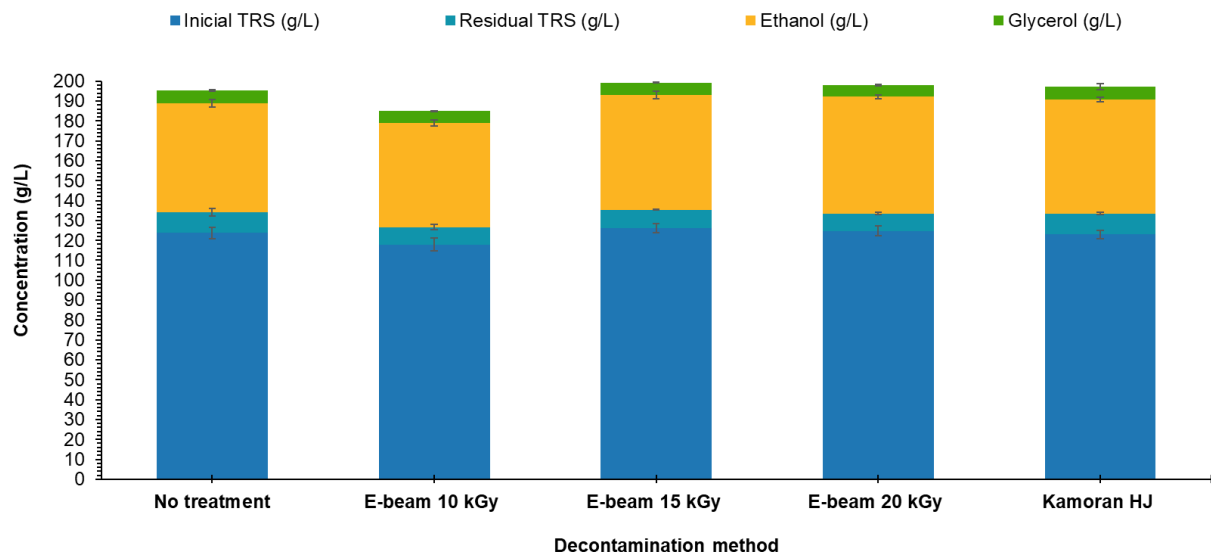


Figure 28 - Effects of mixed wort decontamination in the ethanol, glycerol, and residual TRS obtained in the fermented wort versus the initial TRS

As discussed before, glycerol is an important by-product that plays a critical role in regulating osmotic stress on yeast cells<sup>88,89</sup>. In this experiment, the results showed that the highest difference detected in glycerol content was  $0.6\text{g.L}^{-1}$  between the samples. As illustrated in Figure 28, the concentrations of glycerol remained within the specified range for all conditions tested (where around 10% of the initial total reducing sugars)<sup>88</sup>, indicating that the changes in the mixed wort resulting from the electron beam and Kamoran HJ treatments did not interfere with the osmotic stress experienced by the yeast during fermentation. Consequently, these findings suggest that both the electron beam and Kamoran HJ treatments do not have a significant

impact on glycerol production during alcoholic fermentation, and that the regulation of osmotic stress remains relatively unaffected by these treatments.

Numerically, all decontamination methods applied in mixed wort returned increases on the fermentative parameters. The electron beam at 15kGy, 20kGy and Kamoran HJ were the conditions with more positive impact on the ethanol yield, productivity, and ethanol content. Considering the margin of error, those three conditions presented similar increases in the fermentative parameter.

Interestingly, statistical analysis showed that all conditions had similar results when compared to the control conditions, indicating that the decontamination methods did not have a significant effect on the fermentative parameters. The findings indicate that all tested decontamination methods are suitable for mixed wort fermentation, with no adverse effects on ethanol production. Moreover, the stoichiometric yield for electron beams at 15kGy, 20kGy and Kamoran HJ ranged above the ethanol yield stated in the industry (90-92%), but under the values found in laboratory<sup>91</sup>. The fermentative parameters for the studied decontamination methods are shown in Figure 29.

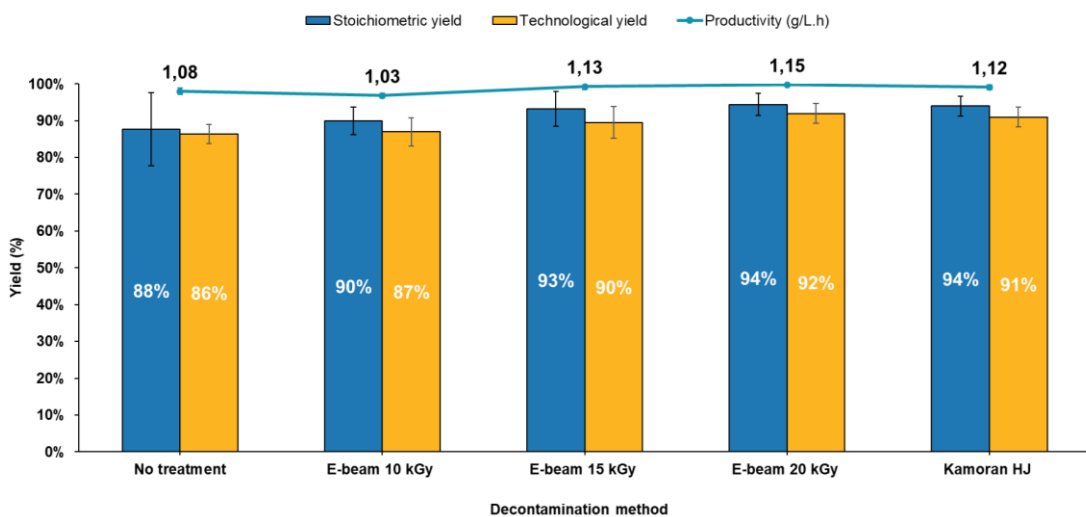


Figure 29 - Effects of mixed wort decontamination in the fermentative parameters. The means of all response variables, including productivity, stoichiometric yield, and technological yield, were statistically comparable to those of the Control sample

According to Lopes<sup>14</sup>, gram-positive bacteria represent 98.52% of the bacterial community in industrial wort. Furthermore, they are responsible for the main reductions in the alcoholic fermentation's yield. This explains the fact that although Kamoran HJ



did not reduce the same microbial contaminant load (CFU.mL<sup>-1</sup>) as effectively as the electron beam at 15kGy and 20kGy, all these three conditions achieved similar ethanol yields. As described by the supplier, Kamoran HJ can control the growth of the main Gram (+) species that contaminate and reduce the alcoholic fermentation's yield<sup>95</sup>.

The alcohol content obtained in the fermentations ranged between 6.6-7.4%. As the initial TRS presented in the mixed wort treated by e-beam at 10kGy was lower than the initial TRS in the other conditions, its ethanol content was the lowest one, but did not differ more than 11%.

It was observed that the decontamination method did not significantly influence the ethanol content acquired, mainly because the microbial contaminants in the mixed wort (10<sup>4</sup>CFU.mL<sup>-1</sup>) was below the prejudicial concentration for alcoholic fermentation (10<sup>7</sup>CFU.mL<sup>-1</sup>)<sup>13,14,15</sup>. Furthermore, when higher than 10<sup>8</sup>CFU.mL<sup>-1</sup>, bacterial contaminations can reduce up to 90% of the ethanol theoretical yield<sup>13,14,15</sup>.

The alcohol content obtained for Ethanol Red fermentation after 43 hours is shown in Figure 30.

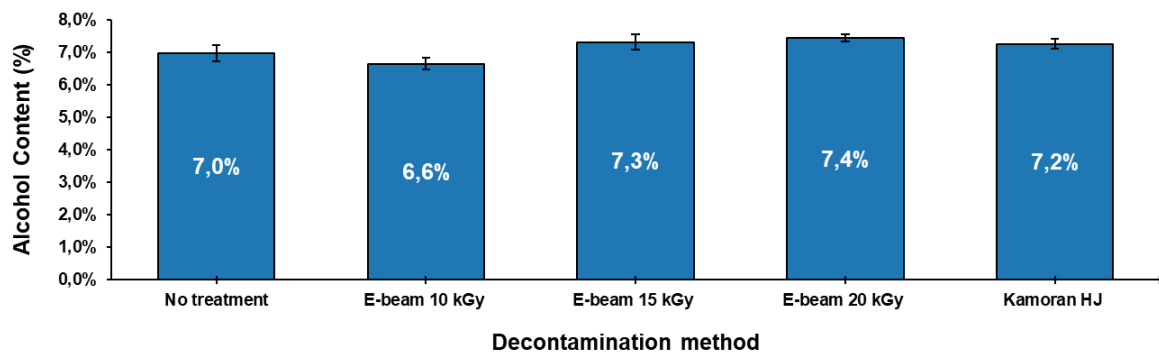


Figure 30 - Effects of mixed wort decontamination in the alcohol content

As previously described, the fermentation's sub-products, namely lactic, acetic, and succinic acids, are utilized to indirectly assess the contaminant's activity in the fermented wort. While the production of lactic and acetic acids is attributed to the metabolic contaminant's activity, succinic acid is produced by the yeast. The measured concentration of succinic acid in the wine was found to be below 1.7g.L<sup>-1</sup>, which is considered the maximum amount produced by yeasts belonging to the *Saccharomyces* genus<sup>96,97</sup>.

The findings of the current investigation reveals that the organic acids present in the fermented mixed wort did not significantly impact the efficiency of alcoholic fermentation. This can be attributed to the fact that the levels of contaminants identified in the wort were below the thresholds that are known to elicit stress responses in yeast cells<sup>95</sup>. Specifically, the concentrations of acetic acid and lactic acid were lower than the range of 0.05-0.1% w.v<sup>-1</sup> and 0.2-0.8% w.v<sup>-1</sup>, respectively<sup>95</sup>. These results suggest that the presence of contaminants in the fermented mixed wort is not likely to pose a substantial risk to the successful completion of the fermentation process. The organic acids identified in the fermented mixed wort are displayed in Figure 31.

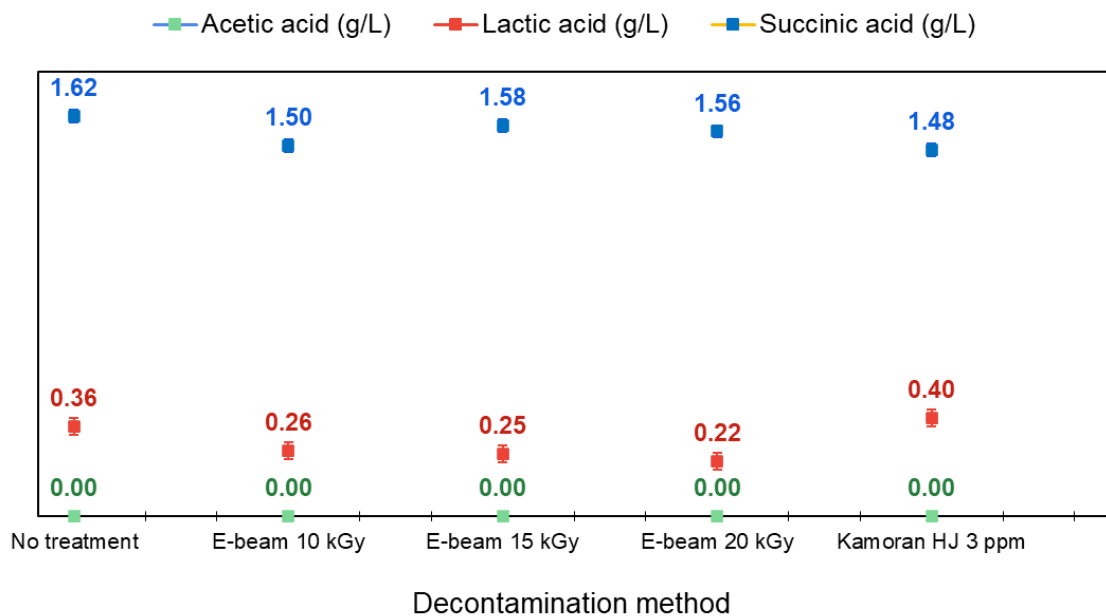


Figure 31 - Effects of mixed wort decontamination in the organic acids (acetic, lactic, and succinic)

In alcoholic fermentation, the presence of mannitol can potentially impact the yield of ethanol and the viability of the yeast cells. In this study, the mannitol concentrations in the fermented mixed wort were measured, and their impact on ethanol yield and cell viability was evaluated. The concentrations of mannitol ranged between 0.07-0.15g.L<sup>-1</sup>, which is below the threshold concentration of 2.5g.L<sup>-1</sup> at which mannitol can potentially interfere with ethanol yield and cell viability<sup>99</sup>.

In this study, the impact of two different decontamination methods, electron beam irradiation and the addition of Kamoran HJ, on yeast viability in alcoholic fermentation was assessed. To this end, yeast cell counts were measured before and

after fermentation using a Neubauer chamber, and the results are presented in Figure 32.

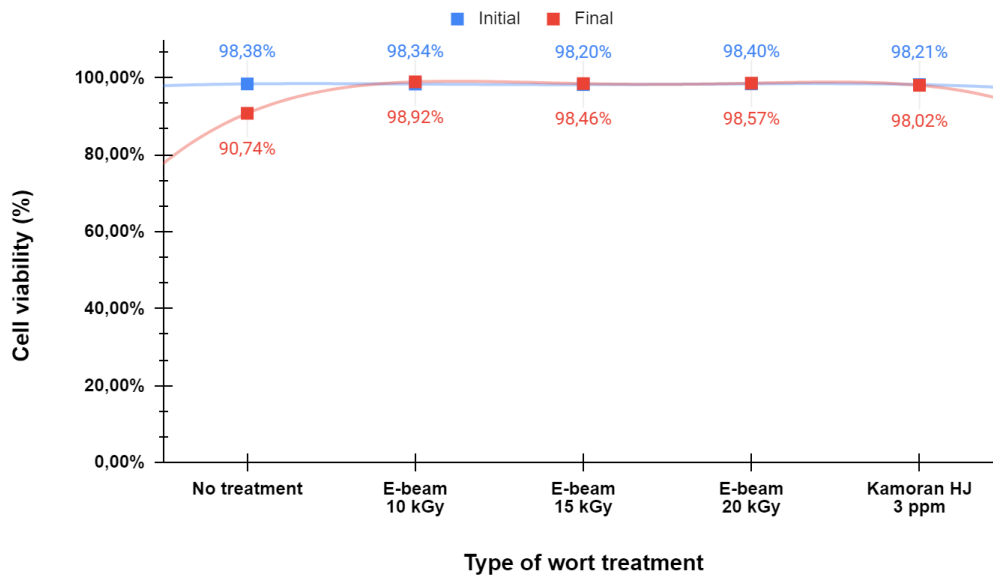


Figure 32 - Effects of mixed wort decontamination in the Ethanol Red's cell viability

The results of this study suggest that electron beam irradiation and Kamoran HJ treatment are effective decontamination methods that do not negatively impact the viability of ethanol-producing yeasts during alcoholic fermentation. Both decontamination methods helped the yeast to maintain the initial viability of ca. 98% until the end of fermentation. It is worth noting that these results are consistent with the supplier's claims that the antibiotic Kamoran HJ's action does not affect ethanol production by the yeast.

Finally, after 43 hours of fermentation, bacterial contamination was measured by analyzing the Petri dishes containing cycloheximide. Notably, no colonies were found, indicating that Ethanol Red was the dominant microorganism acting in the fermentation process. Bacterial contamination is a common issue in alcoholic fermentation that can negatively impact the fermentative parameters. These findings suggest that the decontamination methods used in this study, electron beam irradiation and antibiotic treatment with Kamoran HJ, were effective in reducing bacterial contamination.

### 5.3 Topic IV: impacts of scaling up mixed wort fermentation

In this section, four conditions were selected and tested in benchtop bioreactors based in their improvements in the biomass production, and fermentative parameters. The scaling up was performed with corn and sugarcane mixed wort treated by e-beam at 20kGy, Kamoran HJ 3ppm, integrating supplementation and e-beam 20kGy, and compared with the mixed wort with no treatment (control).

As described in Figure 33, the concentrations of ethanol, total reducing sugars (TRS), and glycerol followed an exponential pattern during the initial 24 hours of fermentation. After 72 hours, more than 95% of the TRS had been consumed, leaving a residual TRS of approximately  $4.77\text{g.L}^{-1} \pm 1.29$ . Glycerol profiles were similar across all conditions, with maximum production observed in the group that received supplementation and e-beam treatment at 20kGy ( $9.12\text{g.L}^{-1} \pm 0.08$ ). At the end of fermentation, average ethanol concentrations were  $68.63 \pm 2.78\text{g.L}^{-1}$ , and fermentative parameters (productivity, ethanol content, stoichiometric and technological yield) were significantly similar across all four conditions.

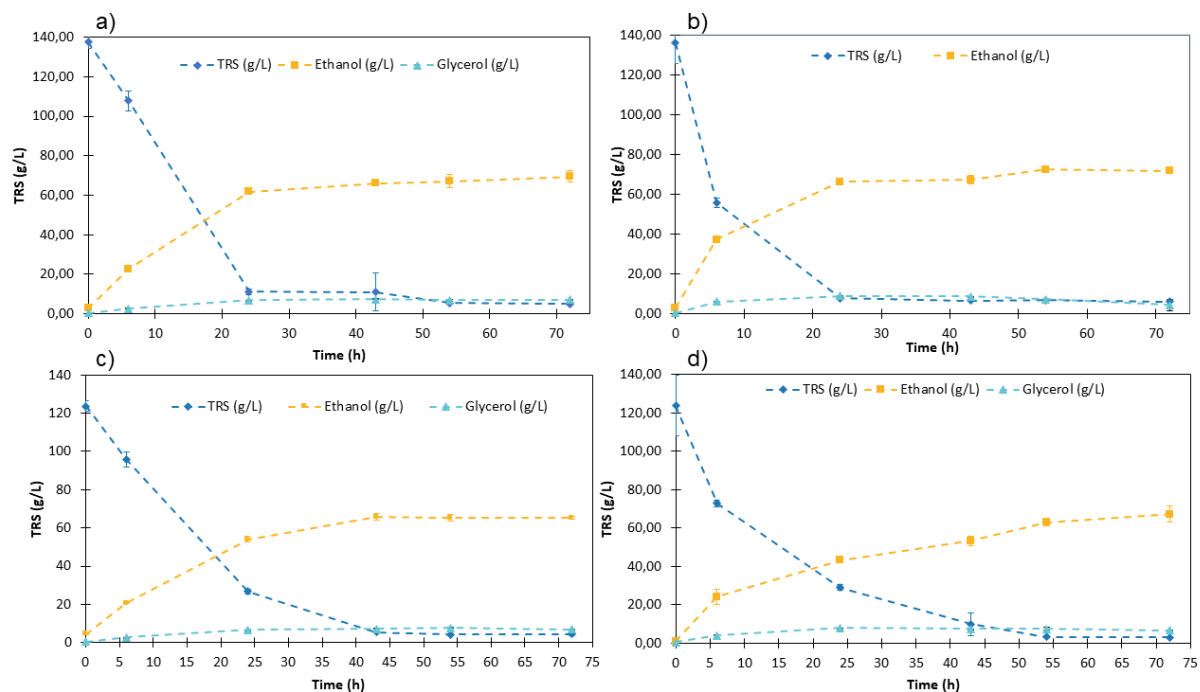


Figure 33 - a) Control, b) Kamoran HJ 3ppm, c) e-beam at 20kGy e d) e-beam 20kGy and supplementation

The alcohol content obtained for all conditions ( $9\% \pm 1\%$ ) was lower than the alcohol content typically detected on an industrial scale, which ranges from 12% to

18% (v.v<sup>-1</sup>)<sup>14</sup>. This disparity can be attributed to several factors, notably variances in the wort preparation process and the concentration of the inoculum added to initiate the fermentations.

After 43 hours, no increase of more than 5% was observed in the organic acids, ethanol, and glycerol concentration in the fermented mixed wort. In these tests, the concentration of mannitol, lactic, acetic, and succinic acid ranged from 0 to 1.6 mg.L<sup>-1</sup>, which indicates a reduced activity of microbiological contaminants during fermentation<sup>95,96,97,99</sup>.

In addition, the synthesis of organic acids can reduce both the pH of the medium and yeast growth. While the optimal pH range for yeast growth can fluctuate between 3.2 and 6.0<sup>105</sup>, the ideal pH for alcoholic fermentation is more limited, falling within the range of 4 to 5<sup>106</sup>. The pH variation during the tests in benchtop bioreactors is described in Figure 34.

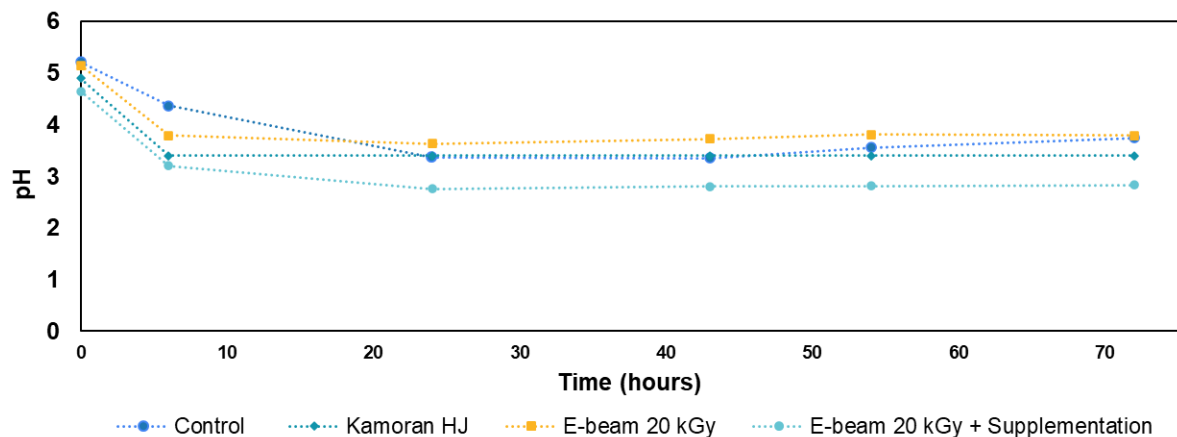


Figure 34 - pH variation during mixed wort fermentations in benchtop bioreactor

The fermentations started with a pH of  $4.98 \pm 0.31$ , and during all tests the pH decreased to  $3.64 \pm 0.21$ , except the condition integrating e-beam 20kGy and supplementation, which ended the process at 2.83, the highest pH variation (39%). This condition that integrated e-beam 20kGy and supplementation likely had a specific combination of factors that contributed to the lower pH. The supplementation could have provided additional nutrients that encouraged microbial growth and metabolism, leading to more pronounced acid production, and pH decrease. Additionally, ammonium sulphate ( $(\text{NH}_4)_2\text{SO}_4$ ) dissociates in water to form ammonium ions ( $\text{NH}_4^+$ ),

and sulphate ions ( $\text{SO}_4^{2-}$ ). Ammonium ions can undergo the process of nitrification, releasing protons ( $\text{H}^+$ ) into the solution, which lowers the pH.

Regardless the chemical stress with pH variation, the cell viability did not significantly differ between all conditions tested. During fermentation, ethanol production can have a negative impact on cell viability. This is because ethanol is toxic to yeast cells and can damage their membranes, leading to cell lysis.

To minimize the negative effects of ethanol on cell viability, it is important to optimize the fermentation conditions. This includes controlling parameters such as temperature, pH, and oxygen levels in the bioreactor, as well as the concentration of nutrients and other additives in the wort. As evidenced in Figure 35, variations in Ethanol Red cell viability did not exceed 5% across the entire fermentation process conducted in bioreactors. Consequently, it was unnecessary to modify any of the above-mentioned parameters to obtain results comparable to those achieved in the tests carried out on conical tubes scale.

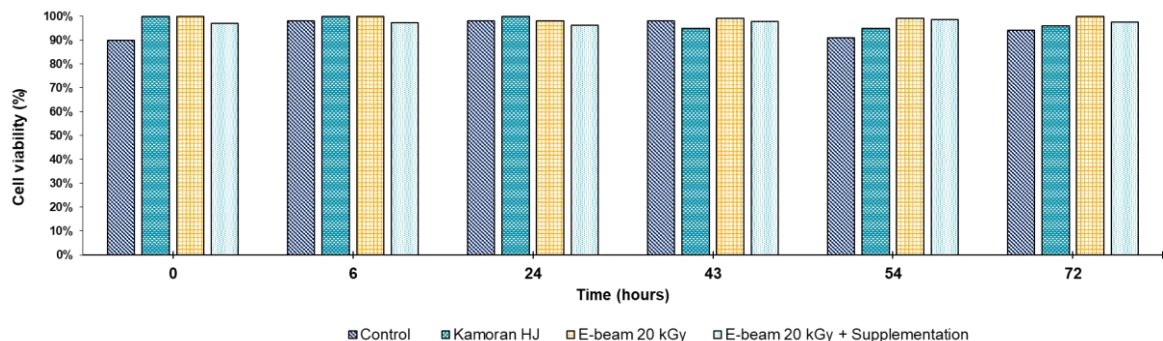


Figure 35 - Cell viability in mixed wort fermentation of Ethanol Red in benchtop bioreactors

During ethanol fermentation, the concentration of yeast cells in the fermentation medium can vary depending on the initial inoculum size, the nutrients availability, the composition of the culture media and other environmental conditions. The Ethanol Red cell concentration throughout the 72 hours of fermentations is shown in the Figure 36 for all conditions tested in the benchtop bioreactor.

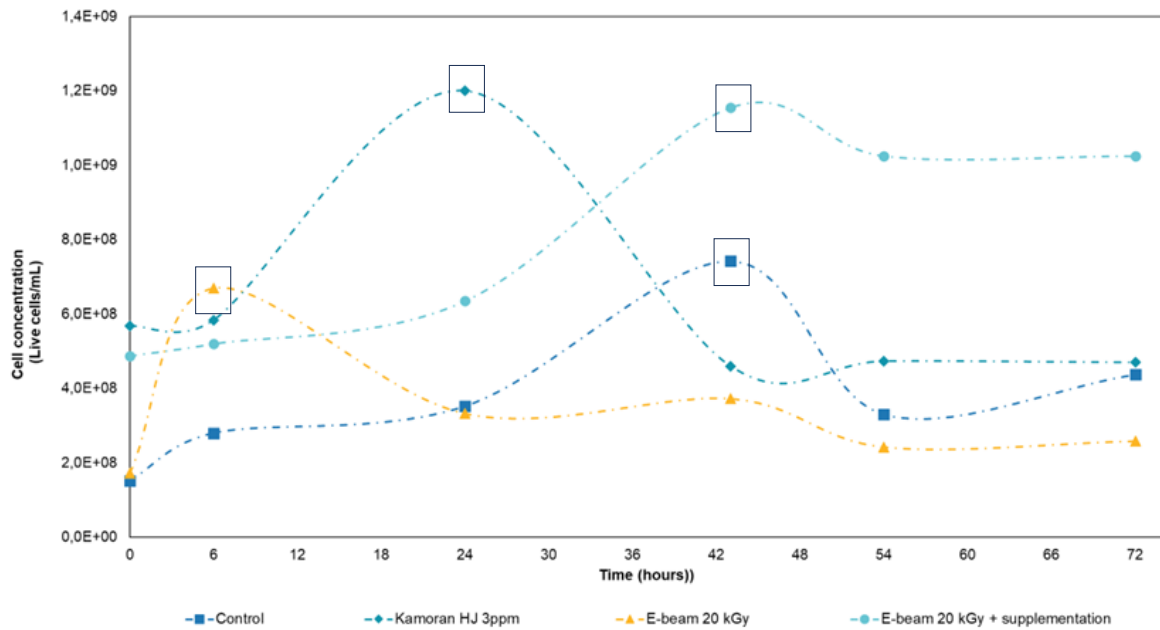


Figure 36 - Ethanol Red cell concentration throughout fermentation process in benchtop bioreactors a) Control, b) Kamoran HJ, c) e-beam at 20kGy, and d) e-beam 20kGy plus supplementation

As seen in Figure 36, Ethanol Red cells treated with e-beam at 20kGy had a shorter lag phase and reached their maximum concentration 37 hours earlier compared to the control condition. Similarly, the use of Kamoran HJ also accelerated biomass production by 19 hours, although to a lesser extent. Analyzing the increase in cell concentration values (live cells.mL<sup>-1</sup>) during fermentation, it was observed that the control condition achieved a cell concentration 3.9 times higher than the initial cell concentration supplied. In a less pronounced manner, the treatments with e-beam, Kamoran HJ, and e-beam at 20kGy plus supplementation showed increases of 2.9, 1.1, and 1.4, respectively.

Additionally, the e-beam treatment at 20kGy plus supplementation resulted in lower values of cell concentration increase and biomass production velocity. This can be attributed to the pH measured at the end of fermentation, which was approximately 2.83. This pH value falls outside the optimal pH range for yeast growth, which is typically between 3.2 and 6.0<sup>105</sup>.

Finally, studies in chemostats provide insights that faster growth rates result in higher maximum cell concentrations, as they can divide more rapidly and reach greater concentrations before facing nutrient or other growth factor limitations<sup>109</sup>.

Nevertheless, the correlation between these two variables can fluctuate depending on the growth conditions. For instance, in the bioreactor experiments, the conditions that hastened biomass production did not yield the highest concentration of cells, which was instead observed when the mixed wort was not treated (control).



## 6 CONCLUSIONS

1. In all tests, the microbial contaminant indicators (acetate, lactate, succinate, and mannitol) were under the prejudicial range for alcoholic fermentation and no bacterial colonies were detected in the fermented wort. Furthermore, the main conclusions about each Topic from this dissertation can be find below.

### Topic I:

2. According to physicochemical characterization of feedstocks, carbon/Nitrogen ratios are above the required levels for ethanol production in mixed wort for a process without yeast recycling;

3. Glucose and sucrose are the main sugars in the corn hydrolysate and in sugarcane syrup, respectively;

4. Regarding minerals, Manganese and Cobalt are below the range proposed in the literature for ethanol production while Phosphorus, Magnesium and Iron exceed the range for both feedstocks.

### Topic II:

5. Although Nitrogen, Cobalt, Sodium and Phosphorus were the nutrients with the highest positive impact on yeast specific growth rate, only Nitrogen, Manganese and Potassium expressed a significative increase in the fermentative parameters;

6. Nitrogen is the nutrient with the highest positive impact on yeast specific growth rate, technological ethanol yield and productivity;

7. Nitrogen supplementation in the mixed wort avoid stuck fermentation performed with yeast concentrations lower than 1% (wet basis);

8. Nitrogen supplementation as ammonium sulphate induces glycerol overproduction. However, further research should be conducted to establish a Nitrogen concentration able to drive the yeast metabolism preferentially for ethanol production rather than biomass production;

9. Among the nutrients assessed, only Iron and Calcium have a negative impact on the yeast specific growth rate and the fermentative parameters.

#### Topic III:

10. Electron beam treatment at 15kGy allows achieving commercial sterilization of mixed wort with up to  $10^4$  CFU.mL<sup>-1</sup> microbial contamination, whereas a 20kGy dose is required to sterilize mixed wort;

11. There is no variation in carbohydrate content due to electron beam treatment until doses of 20kGy;

12. E-beam at 15kGy and 20kGy increases yeast specific growth rate in corn and sugarcane mixed wort fermentation without yeast recycling;

13. Although e-beam at 15kGy, 20kGy, and Kamoran HJ at 3ppm show similar numerical enhancements in the fermentative parameters, these increases are not statistically significant;

14. Both decontamination methods evaluated (e-beam and antibiotic) do not reduce yeast viability;

15. In future research, external contaminants should be added to the mixed wort to investigate the impacts of decontamination methods in conditions with microbial contaminants load higher than  $10^4$ CFU.mL<sup>-1</sup>.

#### Topic IV:

16. Scaling up mixed wort fermentation in benchtop bioreactors do not impact cell viability of Ethanol Red™;

17. As well as the tests in microplate for ELISA, the results from the benchtop bioreactors tests demonstrated that electron beam treatment accelerates the biomass production of Ethanol Red in corn and sugarcane mixed wort;

18. The benchtop bioreactors tests with mixed wort treated by e-beam, antibiotic and integrating e-beam at 20kGy and supplementation do not statistically increase the fermentative parameters.

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