

Sterilisation of Wort from Sugarcane Molasses by Electron Beam for Bioethanol Production

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Abstract

The presence of microbial contamination in the wort during the fermentation process results in damages of billions of dollars per year all around the world and promotes the industry dependence of chemicals and antibiotics to control the contamination. For these reasons, this study aimed to use the electron beam to sterilise wort from sugarcane molasses and investigate its bioethanol fermentation. Five treatments (T0 – T4) were carried out using ionizing doses of radiation through the electron accelerator: (0, 10, 20, 40, and 80 kGy) and a positive control (T5-steam by autoclave) were performed. It was evaluated total mesophiles, total bacteria, sugars, phenolics, flavonoids, 5-hydroxymethylfurfural, and Furfural. After the irradiation process, it was conducted an alcoholic fermentation assay using baker's yeast *Saccharomyces cerevisiae*. It was not observed inversion of sugars and formation of the inhibitory by-products flavonoids, furfural, and 5-hydroxymethylfurfural, with exception of the phenolic compounds. Only T4 and T5 were able to sterilise the wort. However, T3 was able to eliminate >99.99% of the microorganisms. In the fermentation T2 promoted the best ethanol yield and productivity among the irradiated treatments, evidencing the possibility of electron beam use in the wort treatment prior to fermentation which may allow a reduction in losses caused by microbial contamination, besides the possibility of promoting fermentation yield and productivity increase. The operation cost for applying the e-beam with a dose of 20 kGy in a standard ethanol plant was estimated at US\$ 0.014 per m³ of wort.

Introduction

The worldwide demand for pollution reduction and uses of renewable energy has increased in recent years. At the same time, the need to make the best use of natural resources for highly efficient and sustainable biofuels. In this sense, the production of ethanol in Brazil is mainly based on the fermentation of sugarcane using the mesophilic yeast *Saccharomyces cerevisiae* (Ponce et al. 2016). The development of strategies to increase the efficiency and productivity of this microorganism are of great importance.

The efficiency of this biological agent in turning the sugar from the sugarcane juice or molasses into alcohol depends very much on the quality of the wort to be fermented. In industrial production, usually, this substrate is not sterilized prior to the fermentation process, which allows the entry of a large number of microbial contaminants in the process, which negatively affects efficiency and productivity (Amorim et al. 2011; Lopes et al. 2016).

Among the main losses caused by microbial contamination, we can cite formation of acids, increased flocculation, and reduction of yeast viability. The population of contaminating bacteria in fermentation can reach levels higher than 10⁷ cells mL⁻¹, which can lead to a significant reduction in alcoholic yield, up to 55% of the expected theoretical value (Amorim et al. 1981). Moreover, a concentration of 10⁸ cells mL⁻¹ may be responsible for the drop in production of 10 to 30 thousand liters of ethanol in a distillery with a production capacity of one million liters per day (Amorim et al. 2011).

Commonly, the Brazilian sucro-energetic industries perform the acid treatment of yeast cream (Melle-Boinot process), which requires a large volume of sulfuric acid to reduce bacterial contamination (Basso et al. 2008; Costa et al. 2018; da Silva-Neto et al. 2020) This process is repeated countless times throughout the harvest, which last about 200 days a year (Brown et al. 2013). However, it is not completely efficient (Ceccato-Antonini 2018), may select resistant bacteria and, consequently promote the osmotic stress of the yeasts, and as a response, the yeasts produce greater amounts of glycerol, thereby reducing the yield of the fermentation (Basso et al. 2011). Furthermore, wild yeast strains rapidly contaminate the fermentation and after a few cycles, only the wild strains survive the acid treatment (Brown et al. 2013).

Another common way to control bacterial contamination in distilleries is the use of antibiotics, but drug resistance has been a limiting factor in the contamination control efficiency (Muthaiyan et al. 2011).

In this case, the use of sterilized wort for ethanol production may allow the use of selected yeast strains, with desirable and stable characteristics throughout the harvest season. Also, it is possible to reduce costs by eliminating or reducing the use of antibiotics, anti-foaming agents, acid treatment of yeast, and consumption of other inputs. It is also possible to reduce the time of recycling the yeasts in the industrial unit, which increases productivity over time. This means that the process could be optimized, costs reduced and productivity increased (Nolasco Jr 2010).

It should also be noted that some technologies, which are not usual today due to the lack of stability of the fermentation process to ethanol production, may become viable if working under aseptic conditions.

There are several methods that can be used for the sterilization of the wort, the substrate for alcoholic fermentation. However, in industrial plants such as the ethanol industry, where the volume of wort processed is huge, the traditional methods like conventional heat and chemical agents can be very costly. On the other hand, this industry has an energy surplus (Cervi et al. 2019), which can help to circumvent this problem, or rather, is self-sufficient in energy, and part of this energy can be used to generate a source to sterilize the wort.

In this way, Electron beam (e-beam), a type of Ionizing radiation (IR) is a quite effective technology for microorganisms inactivation and generally depends on the dose of radiation applied, where the logarithmic number of microorganisms decreases linearly with increasing dose (Sampa et al. 2007).

Besides, the doses used for sterilization do not produce radioisotopes or radioactive waste, demonstrates benefit over other irradiators such as Cobalt 60 (gamma irradiation), so its residue is the products derived from the radiolysis of water, such as water, hydrogen, and oxygen (Kochetkov et al. 1979; Schwarz 1981).

These radicals interact with the molecules and produce oxidation, reduction, dissociation, and degradation. Besides that, the IR can promote water radiolysis and consequently the destruction of microorganisms, insects, and other parasites (Molins 2001).

It should be noted that the main target of IR is the cells' genetic material DNA and/or RNA. The lesions promoted in the single DNA helix can result in mutation, but a large number of injuries may exceed the repair capacity of the microbial cell resulting in cell death (Stockwell et al. 2017). Other damages in crucial components of the cell such as proteins and lipids can be promoted by IR (Nie et al. 2012).

Withal, e-beam is a very safe method, it is cold, has a high Sterility Assurance Level (SAL), needs a short exposure time and the control parameter of the method is the dose applied. Furthermore, it is an on-off technology that operates with electric power and has a much higher dosing rate than other radiation technologies, such as gamma (γ) and X-rays (Silindir and Özer 2009). One limitation of the technology is that it has low penetrating ability in materials (Lung et al. 2015). But the sugarcane wort can be irradiated in a thin liquid layer at controlled flow (Alcarde et al. 2001).

E-beam is an emerging technology that has been applied to industrial purposes, such as pharmaceutical and medical packing sterilisation, food disinfection and sterilisation (radappertization), and also to the treatment of water, municipal and industrial wastewater (Kurilova et al. 2015; Lung et al. 2015).

For these reasons, it is believed that the use of e-beam is a good strategy for the radappertization of the wort (commercial sterilisation) because allows it to be installed in the production line and to use part of the surplus energy of the industrial plant. Carrying in this way, greater efficiency in the processes of alcoholic fermentation.

Therefore, this study aimed to evaluate the application of the e-beam to control the contamination in wort from sugarcane molasses for alcoholic fermentation and to investigate the yield and productivity of alcoholic fermentation from wort treated with e-beam ionizing radiation.

Material And Methods

Material

The molasses of sugarcane used for the preparation of the wort to the alcoholic fermentation were obtained from a factory in Piracicaba city, São Paulo State, Brazil (22° 43 '31 "S, 47°38' 57" W), followed by physicochemical characterisation and stored in a freezer (-20 ° C).

Wort clarification

The sugarcane molasses used for the preparation of the wort underwent the clarification process following Braga (2006) recommendations with the addition of 2,5 g of $\text{NaH}_2\text{PO}_4 \text{ L}^{-1}$ in boiled molasses. After the addition of the reagent, the molasses was autoclaved and held for 48 hours for further separation of the supernatant from the sedimented material.

At the end of the clarification step, the molasses with an initial concentration of 70 °Brix 9628.75 g L^{-1} of total reducing sugars) was diluted with distilled water until the final concentration of 16,7 °Brix (150 g l^{-1} of total reducing sugars) was obtained.

Preparation of contaminating inoculum and inoculation

For the preparation of the contaminant inoculum, a sample of 10 grams of soil from several points in a cane field was collected, to simulate the groups of contaminating microorganisms, normally, found in the process of alcoholic fermentation in sugarcane mills. This sample was mixed with 90 ml of the clarified molasses, filtered using quantitative filter paper N.640, 125 mm (Hellma®), and placed in a 250 ml Erlenmeyer flask. This Erlenmeyer was maintained at 30 °C, under stirring at 100 RPM, for 24 hours, using a shaker model Minitrons Infors®.

After 24 hours, the inoculum suspension reached 2.01×10^{12} CFU ml^{-1} (Colony Forming Unit) of total mesophile and 1.32×10^{12} CFU ml^{-1} of total bacteria. Then, the inoculum was used to contaminate the wort. The final concentration in the wort was 1×10^7 CFU ml^{-1} of total mesophile.

Treatments

The wort utilized in the investigation was submitted to six treatments. The first treatment, the negative control – without elimination of contaminants microorganisms (T0), four treatments using different ionization radiation doses from electron beam source: 10 kGy (T1), 20kGy (T2), 40 kGy (T3), 80kGy (T4) and one treatment using steam sterilisation – as the positive control (T5), which will be detailed below.

E-beam treatments

The irradiation treatments were performed in the Technological Radiation Center (CTR) of Energy and Nuclear Research Institute (IPEN – CNEN/SP). The samples were irradiated by the Electron Beam Accelerator JOB 188 (Dynamitron®).

The batch irradiation process was chosen, and the wort was added to borosilicate rectangular glass vessels (Pyrex®) and packed with plastic film 0.1 mm. Each vessel received 300 ml of wort obtained from clarified molasses, which corresponds to a height of 4 mm of the sample. For each batch, 4 vessels were irradiated.

The Electron Accelerator was set to energy, width, and current of the electron beam of 2.4×10^{-13} J (1.5 MeV), 0.112 m, and 5.61×10^{-3} A, respectively. The tray speed was 0.112 m s^{-1} , proportional to a dose of 5 kGy per run.

Steam sterilisation treatment

The T5 treatment was performed by autoclaving the clarified molasses at 121 °C, 1 ATM, for 20 minutes in a vertical autoclave (Phoenix®).

Evaluation of the contamination control

The evaluation of the growth of total bacteria and total mesophiles was performed by the logarithmic variation in the number of CFU: $\text{Log}(\text{CFU ml}^{-1} + 1)$. It was added 1 to the CFU because of the treatments that presented 0 CFU due to the result of $\text{Log} 0$ is an undefined value.

The D_{10} (Required dose to destroy 90% of the population or 1 log) for total bacteria and total mesophile were calculated in kGy according to Equation 1, where N_0 is the initial CFU ml^{-1} and N_{final} is the CFU ml^{-1} after irradiation.

$$D_{10} = \frac{\text{applied dose}}{(\text{Log}_{10} N_0 - \text{Log}_{10} N_{\text{final}})}$$

Eq. 1

The efficiency control of the microorganisms was calculated according to Equation 2.

$$\text{Efficiency of control (\%)} = \left(\frac{N_0 - N_{\text{final}}}{N_0} \right) \times 100$$

Eq. 2

Chemical and microbiological analyses

After the treatments, the materials underwent chemical and microbiological analyses described below.

Total mesophile and total bacteria

For the enumeration of the microbial contamination levels, the total mesophile and total bacteria were measured by taken aseptically a sample of 1 ml of the wort and serial diluted with 9 ml of saline solution (0.85% NaCl in distilled water).

After the serial dilution, the samples were pour plated in Plate Count Agar (PCA) in order to determine the total mesophile and, in PCA with 10 mg l⁻¹ to determine the total bacteria. All plating was performed in triplicate with incubation at 30 °C for 48 hours.

Sugars, glycerol and mannitol

Glycerol, mannitol and the sugars sucrose, glucose and fructose were measured by ion chromatography, following the method described by the manufacturer (Eith et al. 2006).

It was used the ion chromatograph 930 Compact IC Metrohm® equipped with the column 1 Metrosep Carb 150 / 4.0, at 35 °C with eluent solution of 200 mM sodium hydroxide and the flow was set to 0.5 ml min⁻¹. The wort samples were diluted 200 times and the wine samples were diluted 50 times with ultrapure water. After that, the samples were filtered with 0.45 µm cellulose acetate filter. All the samples were measured in triplicates and the volume of sample injected was 20 µL.

Furfural and 5-hidroximetylfurfural

Furfural and 5-hidroximetylfurfural (5-HMF) were measured by gas chromatography with Flame Ionization Detector (FID), following the method 72 described by U S Department of Labor Occupational Administration Safety and Health (1988).

It was used the gas chromatograph GC-FID Shimadzu equipped with the column ZB-5 Phenomenex® 60 m x 0.32 mm x 1.00µm. The chromatographic conditions were injector temperature 170 °C, injection mode split, carrier gas N₂, flow rate 6.0 ml min⁻¹ (carrier gas), split ratio 8.0, column temperature 50 °C for 5 min, then temperature program to 150 °C at 5 °C min⁻¹, detector temperature 200 °C.

The wort samples were filtered with 0.45 µm cellulose acetate filter and the injection volume of the sample was 2.0 µL.

Total phenolics

The total phenolics of the wort samples were determined according to the Folin-Ciocalteu method according to Julkuentiitto (1985) with modifications. The procedure consisted of adding 1000 µL of the ample (or standard for calibration curve) and 500 µL of Folin Ciocalteu reagent (10% v/v) to a test tube, wait 40 minutes and then add 2500 µL of Na₂CO₃ (20 % m/v). After that, the absorbance was measured at 725nm in a quartz cuvette on the spectrophotometer (UV mini-1240 Shimadzu®).

The standard curve was prepared with 6 points of tannic acid (0, 4, 8, 12, 16, 20, 25 $\mu\text{g ml}^{-1}$) with 3 repetitions at each point. The samples were diluted with ultrapure water 25 times and also measured in triplicate.

Total flavonoids

The total flavonoids of the worst samples were measured according to Mabry et al. (2012) with modifications. Thus, 4.3 ml of ethanol (70% v v⁻¹), 100 μL of 2% AlCl_3 (w v⁻¹, solution in methanol), 100 μL of sodium acetate 1M and 500 μL of the sample (or standard for calibration curve) were added to a test tube and kept at rest for 40 minutes. Then, the absorbance was measured at 415nm in a quartz cuvette on a spectrophotometer (UV mini-1240 Shimadzu®).

The standard curve was prepared with 8 points of rutin solution (0, 1, 2, 4, 8, 16 $\mu\text{g ml}^{-1}$) with 3 repetitions each point. The samples were diluted with ethanol (70% v/v) 50 times and also measured in triplicate.

Fermentation

The worts from all treatments, except T4 (80 kGy), were submitted to the fermentation process. We chose the treatments with the first three doses for the fermentation because we understood that these would meet the necessary conditions to reduce microbial contamination and achieve high productivity and fermentative yield glimpsing the industrial application.

The fermentation process was conducted in 500 ml Erlenmeyer flasks containing 200 ml of wort, with 16.7° Brix, 150 g L⁻¹ of total reducing sugars, and 3% of the dry yeast *Saccharomyces cerevisiae* "Fleischmann" (AB Brasil Indústria e Comércio de Alimentos Ltda).

The fermentation process was conducted with 5 replicates (reactors) per treatment, at 30 °C, under 100 RPM stirring (Minitron Shaker – Infors HT®). The process was monitored through the losses of CO₂ from the reactors during the fermentation. At the end of the fermentation, yeast cell viability and total bacteria were measured, and the fermented wort was centrifuged at 3738.8 g (Thermo Fischer Scientific Sorval ST 40R® centrifuge), at 10 °C for 10 minutes. Then, the wine was immediately frozen (-20 °C) for further analyses.

The chemical and microbiological analyses of the wines were: total bacteria, sugars, glycerol and mannitol, as previously described and also yeast cell viability, yeast cell biomass and alcohol content, as described below.

Yeast cell viability

The yeast cell viability was determined according to Pierce (1970) by the differential staining of living and dead cells using 0.1% methylene blue solution and observation on an optical microscope (Nikon®). This analysis was performed at the beginning and the end of the fermentation.

The samples were prepared with the dilution of 500 μL of the wine 10 times with distilled water. After that, 300 μL of the suspension was transferred to a tube with 300 μL of 0.1% methylene blue solution and homogenized. Then, 10 μL of the suspension was transferred to a Neubauer chamber followed by the observation on a microscope (400x).

Yeast cell biomass

The yeast cell biomass was determined by the wet weight according to Zago et al. (1996). For this, the fermented wort was centrifuged at 3738.8 g (Thermo Fischer Scientific Sorval ST 40R® centrifuge), at 10 °C for 10 minutes. Then, the pellet mass was measured on a semi-analytical scale (BL320H Shimadzu®) and compared with the initial mass of the sample.

Alcohol content

The alcohol content of the wine was measured by the distillation of 25 ml of sample in a micro-distiller (MA 012/1 Marconi®) followed by density measurement using a Digital Densimeter (EDM 4000 Schmidt Haensch®) at 20 ± 0.05 °C, according to Zago et al. (1996).

The density value of the solution was used to calculate the alcohol concentration of the sample by converting the read density in %m m⁻¹ using a conversion table at 20 °C/ 20 °C.

Fermentation yield and productivity

The yield (practical) was calculated based on the volume of ethanol obtained from 100g of sugars supplied in the substrate according to Equation 3.

$$Yield = \left(\frac{ml \text{ of final ethanol}}{g \text{ of sugars supplied}} \right) \times 100$$

Eq. 3

The productivity was calculated according to Equation 4 based on the alcohol content at the end of the fermentation t and the fermentation time. The productivity was expressed in grams of ethanol per hour (g l⁻¹ h⁻¹)

$$Productivity = \frac{\text{ethanol concentration in the wine } g\ l^{-1}}{\text{fermentation time}(h)}$$

Eq. 4

Electrical consumption estimation

For the estimation of the energy cost to operate the electron accelerator used in this study, initially, it was calculated the energy consumption in KWh of the electron beam operating with a voltage of 1.5×10^6 V and electric current of 5.61×10^{-3} A for 1 hour. This is the power required to irradiate a sample with a dose of 5kGy approximately. The total energy consumed by the accelerator peripherals such as cooling system, vacuum system, and compressed air was determined through the nominal values of the power supplied by the equipment manufacturers.

Regarding the cost of energy (US \$ / MWh), since the sucroenergetic industry is also an electricity producer, which besides being self-sufficient, is still an exporter of the surplus energy produced, it was considered that the energy consumption needed for the accelerator would no longer be commercialized. Therefore, the value of MWh was equivalent to the average amount that would be paid to the ethanol-producing industry, according to the electric energy commercialization contracts of the Brazilian Electricity Regulatory Agency (ANEEL). For this calculation, it was considered that the amount paid for 1 MWh of the excess energy produced by burning biomass (sugarcane bagasse and wood chips) by ethanol-producing plants in Brazil through public auctions held by ANEEL for energy distributors (CCEE 2021).

According to the auctions held in March / 2016 and April / 2017 (energy supplied respectively in 2020 and 2021), the average amount paid for each MWh of energy was US\$ 42.50 or US \$ 0.0425 / kWh (ANEEL 2021). The dollar rate (US \$ 1.00 = R \$ 5.53) was consulted on 3/21/2021 on the website of the Central Bank of Brazil (BCB 2021).

Experimental design and statistical analyses

The experimental design was entirely randomized with six treatments and five replicates per treatment.

The results were submitted to analysis of variance (ANOVA) by the F test and the averages compared in the Tukey test at the significance level of 5% ($p \leq 0.05$). The statistical analyses were performed using SISVAR 5.6 software (Ferreira 2014).

Results And Discussion

To meet the greenhouse gases emission reduction targets the demand for renewable biofuels has increased. For this, efforts must be made to increase industrial productivity, and for that, better control of microbiological contamination is highly necessary.

The yeast cells reuse during the season countless times can influence the contamination level of the must by bacteria and wild yeasts (Lopes et al. 2016, Brexó and Sant'Ana 2017). The development and predominance of wild strains of yeasts is undesirable for the process due to the lower productivity, flocculation, foaming, and biofilm formation produced by these microorganisms (Beckner et al. 2011, Della-Bianca and Gombert 2013, Della-Bianca et al. 2013). These drawbacks increase the use of antifoam, acids and antibiotics in the industry plant (Brexó and Sant'Ana 2017).

The samples of wort submitted to the treatments T0, T1, T2, T3, T4 and T5 presented 7.00, 3.72, 3.31, 2.41, 0.00 and 0.00 Log (CFU +1) ml⁻¹ ($p < 0.05$) of total mesophiles, respectively (Fig. 1). These results correspond to an efficiency control of the microorganism of 99.94%, 99.97%, >99.99%, >99.99%, 100% and 100% ($p < 0.05$) for T1, T2, T3, T4 and T5, respectively.

For total bacteria, T0, T1, T2, T3, T4 and T5 presented 5.00, 2.33, 3.22, 2.33, 0.00 and 0.00 Log (CFU +1) ml⁻¹, respectively. These results correspond to an efficiency of control of 99.99%, 99.98%, >99.99%, 100% and 100% for T1, T2, T3, T4 and T5, respectively.

Therefore, the microbial contamination of the worst decreased as the irradiation dose increased, in accordance with Sampa et al. (2007) study.

Only treatments T4 (80 kGy) and T5 (steam - positive control) were able to sterilise the must. However, T3 (40 kGy) was sufficient for 4.59-log reduction of total mesophiles and 2.67-log reduction of total bacteria, corresponding to inactivation of more than 99.99% of the microorganisms present in the must.

In a study carried out by Nobre et al. (2007) when submitting sugarcane juice to treatment with ionizing radiation ($g - Co^{60}$), the dose of 15 kGy was not enough to fully inactivate the *Bacillus subtilis* culture, but achieved a reduction of more than 99.9% for these bacteria. In the present study, inactivation of total bacteria higher than 99.9% was achieved in the dose of 10 kGy. Similar results were observed for total mesophiles (Fig. 1). Furthermore, the study conducted by Nobre et al (2007) used pure cultures of bacteria while the present study used the total microbiota from a sugarcane field.

Most of the literature about microbial radioresistance is based on reports of experiments typically involving pure cultures grown under near-optimal conditions (Shuryak 2019). In this study, we used the microbiota from the soil of a sugarcane field since it is known that present many microorganisms groups in different levels. Also, other authors have reported that bacterial contamination in alcoholic fermentation is mostly from the sugarcane field soil (Gallo 1989, Figueiredo et al. 2008, Costa et al. 2015).

A study carried out by Costa et al. (2015) assessing microbial diversity at different stages of sugarcane ethanol production identified 22 archaeal groups, 203 fungi groups and 355 bacterial groups. The authors also mentioned that the microbial contamination increases through the processes in the ethanol plant and are mostly from the feedstock and soil impurities.

Many microorganisms in soil are organic matter decomposers and also opportunistic plant/animals pathogens (Diezmann and Dietrich 2009, Sykes et al. 2014). This way of living requires being able to tolerate and possibly exploit the oxidizing compounds used as a defence mechanism by their hosts (Heller and Tudzynski 2011) this may justify the high radiotolerance of some soil microorganisms.

Also, some microorganisms can synthesize antioxidant compounds and pigments that aid in radioprotection (Kim et al. 2007), such as vitamin C (Mao et al. 2006), carotenoids (Parvathy 1983, Jain et al. 2015) and flavonoids (Molins 2001, Shuryak et al. 2017, Shuryak 2019). These compounds are commonly found in sugarcane juice (Abbas et al. 2014) and also present in the sugarcane molasses, the raw material used in this study (Table 1).

The D_{10} (The required dose to destroy 90% of the population) for total mesophiles was 3.06 kGy, whereas for total bacteria it was 4.81 kGy. Bacteria (prokaryotic) are more radioresistant than other microorganisms such as fungi and viruses, so it is justified that the total bacteria D_{10} is higher than the total mesophiles. In addition, the values found are in accordance with the literature, which states that fungi and bacterial spores present D_{10} values between 1 and 10 kGy (Confalonieri and Sommer 2011, Jung et al. 2017, Shuryak et al. 2017)

Other studies report radioresistant microorganisms like fungi that present chronic and acute radioresistance (D_{10} from 0.1 to 6.5 kGy) (Shuryak et al. 2017), bacteria, such as *Deinococcus radiodurans* capable of withstanding high doses of radiation (D_{10} of 16 kGy) (Omelchenko et al. 2005) and ability to reconstruct the functional genome (Confalonieri and Sommer 2011), and also archaea, such as *Thermococcus gammatolerans* sp. nov., which was isolated after exposure of 30 kGy (g-radiation) (Jolivet et al. 2003).

Moreover, *Lactobacillus plantarum*, one of the major contaminants of alcoholic fermentation (Dong et al. 2015, Dellias et al. 2018), is described as a chronic and acute radioresistant microorganism (Daly et al. 2004, Shuryak et al. 2017).

Most bacterial contaminants are found in the *Lactobacillus* genera (Bonatelli et al. 2017), especially lactic acid bacteria (LAB), like *L. plantarum*, which are responsible for reducing yeast cell viability due to the competition for nutrients and the production of toxic compounds, such as lactic and acetic acids during the fermentation (Narendranath et al. 1997, Costa et al. 2008).

In general, ethanol plants use antibiotics in order to control bacterial contamination. However, in some cases, does not prevent Lactobacilli infections recurrence, since these microorganisms can form biofilm, which is tolerant to the high concentration of the antibiotics and cleaning (Dellias et al. 2018, Saunders et al. 2019).

The large-scale use of antibiotic can induce bacterial resistance (Carvalho et al. 2020). Also, antibiotic residues such as virginiamycin can be found in distillers dried grain (DDG), from bioethanol fermentation of corn, which is utilized for animals feed (Bischoff et al. 2016). Regarding sugarcane bioethanol, there is a concern about antibiotic resistance in microorganisms that may be discharged into the environment through the fertigation using vinasse, the liquid waste obtained from the distillation of the wine (Mendonça et al. 2016). Furthermore, the presence of antibiotic in the vinasse can negatively affect its anaerobic digestion for the production of biogas through the inhibition of acetogenic bacteria and methanogenic archaea (Sanz et al. 1996) and reduce the potential to use vinasse to produce other products.

Therefore, a more efficient disinfection process is needed, such as ionizing radiation (IR). However, the use of IR may promote the formation of inhibitors by-products from sugar degradation (Molins 2001).

In our study, it was not observed formation and alteration in the concentration of the inhibitors flavonoids, furfural and 5-HMF ($p > 0.05$) in any condition of treatment evaluated (Table 1). Such compounds are generally produced from sugar degradation, especially in thermal conditions (Molins 2001, Eggleston and Amorim 2006, Chi et al. 2019). However, it did not occur in this study, including in the steam treatment (T5).

The aldehydes like furfural and HMF may inhibit key enzymes intervening in the rate of protein synthesis of the central metabolism of the yeasts affecting negatively the growth and fermentation (Cabañas et al. 2019). Because of that, the presence of these compounds is highly unwanted in the fermentation substrate.

On the other hand, it was observed a gradual increase of phenolic compounds levels (6%, 9.4%, 17.8% and 19.8%, for T1, T2, T3 and T4, respectively) according to the radiation dose applied (Fig. 2). The steam treatment (T5) presented a significantly lower ($p < 0.05$) concentration of phenolic compounds than 40 and 80 kGy, but it was statistically equal to treatments 10 and 20 kGy.

According to Rasmussen et al. (2014), the degradation of carbohydrates, especially D-glucose, D-xylose and L-arabinose can be related to the production of compounds such as phenolics. These compounds have been considered biocatalyst inhibitors (Chi et al. 2019), and their gradual production according to e-beam dose increase was also observed in Lima et al. (2016) study. But, in our work, the presence of phenolics did not inhibit yeast cell viability and biomass production ($p > 0.05$) in any treatment during the fermentation (Table 3).

In a study conducted by (Martín et al. 2007) using sugarcane bagasse hydrolysate a concentration of 2100 $\mu\text{g ml}^{-1}$ of phenolic compounds was responsible for the yeast (*S. cerevisiae*) inhibition and consequently, poor fermentability. In the same study using an adapted strain of the same yeast it

was observed higher ethanol yield on total sugar after 24 h (0.38 g g^{-1}) than the non-adapted yeast (0.18 g g^{-1}) in wort with $1400 \mu\text{g ml}^{-1}$ of phenolic compounds. In our study, although the yeast has not been adapted to inhibitory toxins, the concentration of phenolics was below $1089.38 \mu\text{g ml}^{-1}$ (Table 1) in all fermented treatments, which probably reflected *the S. cerevisiae* tolerance to these compounds.

In addition to the low formation of inhibitors, it was not observed significant inversion of TRS (Total Reducing Sugars) in all treatments ($p>0.05$) (Table 2). Otherwise, Lima et al. (2016) observed significant ($p<0.05$) TRS inversion in sugarcane juice irradiate with 20 kGy e-beam dose.

The fact that in our study the reduction in the concentration of TRS was not observed is interesting because low sugar degradation is essential in a decontamination method aiming no decrease in the ethanol yield due to the sugars degradation (Alcarde et al. 2000, 2003).

Regarding sucrose, it was observed a decrease in steam treatment ($p<0.05$). However, there was no decrease in concentration ($p>0.05$) in the irradiated treatments (Fig. 3). In a study by Podadera (2007), using electron beam to sterilise invert sugar syrup, it was observed a significant decrease ($p<0.05$) in sucrose concentrations between the control, 5, 10 and 30 kGy samples. Moreover, glucose and fructose concentrations increased significantly ($p<0.05$). Which indicated the degradation of the disaccharide with the breakdown of the glycosidic bond and formation of the reducing sugars glucose and fructose.

At the end of the fermentation, sucrose was not detected in any treatment, in addition, the residual sugars glucose and fructose presented low concentrations ($<0.05\%$) in all treatments ($p>0.05$), evidencing efficient consumption of sugars by yeasts or other microorganisms during the fermentation process (Table 3).

The concentration of glycerol was similar in the wine from all treatments, approximately 15 grams per liters ($p>0.05$). Bai et al. (2008) indicate that during the fermentation commonly a level of about 1% (w/v, 10g l^{-1}) of glycerol is produced.

The high concentration of glycerol in wine can be an indicator of the yeast response to the adversity. High sugar values lead to high concentrations of glycerol in the must, due to the increase in osmotic pressure (Ponce et al. 2016), as well as the presence of bacterial contamination (Li et al. 2009).

There was bacterial contamination in the wine of all treatments. However, the control treatment presented a higher value ($p<0.05$) of $5.55 \log (\text{CFU ml}^{-1}+1)$. The presence of bacteria in all the treatments may be due to the contamination during the experiment sampling and poor asepsis.

As well as the high bacterial contamination, the control treatment (T0) presented a higher concentration of mannitol (0.41g l^{-1}) when compared with other treatments ($p<0.05$). Mannitol is a sensitive indicator of contamination and its presence is an indication of the enzymatic dehydrogenation of fructose carried out exclusively by bacteria (Eggleston et al. 2007).

The T3 wine exhibit the lowest concentration of mannitol (0.33 g l^{-1}) when compared with the other treatments ($p<0.05$). This was expected because among the treatments submitted to fermentation T3 presented the greatest contamination control. This result is remarkably interesting because according to Eggleston et al. (2007) high concentrations of mannitol may promote yeast flocculation and reduce the efficiency and productivity of the fermentation. The authors also described that a concentration around 6 g l^{-1} of mannitol can cause a decrease of 4% in ethanol yield.

The control (T0) showed the lowest yield (88%) of the treatments. It is justified due to high bacterial contamination in wine ($5.55 \log$) and conversion of sugars into metabolites such as glycerol and mannitol (Table 3). The highest fermentation yield was reached in the steam treatment (T5) with 95% ($p<0.05$). Right below, the treatments 20 kGy (T2) and 40 kGy (T3) presented 93% yield ($p>0.05$). Higher than usually fed-batch industrial fermentations with 87% average of fermentation using molasses as raw material (Andrietta and Maugeri 1994, Viegas et al. 2002) and also greater than the yield described by Alcarde et al. (2001) which achieved 90.56% in the fermentation of sugarcane juice treated with 10 kGy (g radiation).

There are great importance and interest in increasing the yield of industrial fermentation. A yield of 93% could be responsible for a significant increase in ethanol production and, consequently, in the revenue of the industrial plant.

Regarding ethanol productivity, it decreased with the contamination whereas the control treatment showed the lowest value of $0.85 \text{ g l}^{-1}\text{h}^{-1}$ (Table 3). The highest productivity has been achieved in steam treatment ($0.91 \text{ g l}^{-1}\text{h}^{-1}$) followed by 10 and 20 kGy treatments with $0.89 \text{ g l}^{-1}\text{h}^{-1}$ in both ($p>0.05$) and 40 kGy treatment with $0.87 \text{ g l}^{-1}\text{h}^{-1}$ ($p<0.05$). Thus, the treatment of 20 kGy showed better productivity ($p<0.05$) and the same yield as treatment 40kGy, which requires more energy.

Due to that, the dose of 20 kGy is the most recommended as it requires less energy consumption to allow the results with very positive response in the control of the contamination and on the fermentation process.

It is believed that greater changes in the fermentative behaviour of the irradiated wort could have been observed if consecutive fermentative recycles and acid treatment of yeast from the control treatment had been carried out. Since the microbial contamination tends to increase throughout the fermentative recycles during the harvest season, as described in the literature (Ceccato-Antonini 2018).

It is also necessary to study more the application of this technology, as well as the increase of the scale and the economic viability.

In our work, the electrical energy consumption to operate the electron accelerator at full power in one hour of use was approximately 150 kWh. Only the electron beam is responsible for 25% (37.5 kWh) of this total. The cooling system, vacuum system, compressed air and other devices consumed the remaining 75% (112.5 kWh). In this case, the cost was US\$ 6.43 per hour.

For the present case, the electron beam was not used at its maximum power. Due to this, for each hour of use of the accelerator, the electrical energy consumption was 122 kWh, of which 113.58 kWh were consumed by the devices of each system mentioned above. Only 8.42 kWh was consumed by the electron beam. The cost was about US\$ 5.23 per hour of operation.

Table 4 presents the operating cost, taking into account only the energy consumption of the electron accelerator for each treatment. Δt is the processing time (or sterilisation) of the samples by e-beam. Their values were obtained considering the conveyor speed of 0.112 m s^{-1} and the linear length of two aligned trays equal to 0.40 m.

Therefore, operating at a dose of 20 kGy, the energy consumption by the electron accelerator is estimated at 146.18 kWh (33.68 kWh) consumed by the electron beam and the rest by the peripheral equipment. The cost of each hour of operation of the accelerator is estimated at US\$ 6.27. Considering that in the ethanol-producing plants in Brazil an average of 450 m^3 of wort is produced per hour, the estimated cost of processing 1 m^3 of this material is US\$0.014.

It is important to note that the sugarcane mills can process 1 m^3 of the wort in a short time. Furthermore, the e-beam technology is very fast, and, in a few seconds, the desired result in microbial control can be achieved. This allows the treatment of large volumes of wort in a short time, which facilitates this process implementation in large industries.

It should be mentioned that apart from sterilisation of the wort, to have success in the contaminating control in the ethanol industry it is necessary an adequate system to cleaning fermenters, pipeline, centrifuges, valves and other compartments used to transport or store wine, yeast cream and wort.

With that, the use of e-beam for sterilisation of the substrate could also make it possible to use more productive yeast strains in the fermentation, such as the thermotolerant strains of *S. cerevisiae* described by Pattanakittivorakul et al. (2019), which show extremely high ethanol production at $40 \text{ }^\circ\text{C}$, in addition to tolerating high gravity fermentation and high concentrations of furfural, HMF and acetic acid.

It should be noted that the e-beam could also be applied in other processes within the industry, as in the pretreatment of biomass for the production of second-generation ethanol (Postek et al. 2018). The biomass irradiation can facilitate enzymatic hydrolysis with lower temperatures and minimal formation of inhibitory by-products when compared to conventional pretreatments (Singh et al. 2016).

Conclusion

The dose of 80 kGy was sufficient for sterilisation of the wort from molasses. Also, the lower dose tested of 10 kGy were able to reduce more than 99.9% of the microbial contamination present in the substrate. The treatment of 20 kGy showed the best yield and ethanol productivity among the irradiated worts, evidencing the possibility of applying the e-beam in the treatment of wort for fermentation, which may allow reduction in losses caused by microbial contamination, besides the possibility of promoting fermentation yield and productivity increase.

Declarations

Ethical approval No ethical approval was required, as human participants or animals were not involved in this research.

Consent to participate not applicable.

Consent to publish All authors have read and agreed to the published version of the manuscript.

Authors contributions Conceptualization: RP, EA, VA, and AS, Methodology: RP, EA, MP, AP, and LA, Formal analysis and investigation: RP, EA, and MP, Resources: VA and AS, Writing – review and editing: RP and AS, Supervision: AS and VA, Funding acquisition: AS.

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Competing Interests The authors declare no competing interests.

Availability of data and material not applicable

Code availability not applicable

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Tables

Table 1. Chemical determinations of inhibitory by-products in the sugarcane molasses wort after treatments

Treatment	Flavonoids	Phenolics	Furfural	5-HMF
	$\mu\text{g ml}^{-1}$			
T0 - Control	26.74 \pm 1.57 ^a	925.27 \pm 13.72 ^c	0.61 \pm 0.00 ^a	0.44 \pm 0.00 ^a
T1 - 10 kGy	23.47 \pm 5.22 ^a	980.68 \pm 11.74 ^b	0.64 \pm 0.02 ^a	0.44 \pm 0.02 ^a
T2 - 20 kGy	25.18 \pm 7.92 ^a	1012.64 \pm 16.20 ^b	0.62 \pm 0.02 ^a	0.44 \pm 0.02 ^a
T3 - 40 kGy	25.44 \pm 1.11 ^a	1089.38 \pm 9.94 ^a	0.62 \pm 0.01 ^a	0.43 \pm 0.01 ^a
T4 - 80 kGy	27.99 \pm 4.61 ^a	1108.79 \pm 28.00 ^a	0.63 \pm 0.01 ^a	0.45 \pm 0.01 ^a
T5 - Steam	32.55 \pm 4.75 ^a	993.77 \pm 27.33 ^b	0.64 \pm 0.01 ^a	0.42 \pm 0.01 ^a
C.V.	18.6	1.68	2.22	3.54

Averages of $n=5$ \pm standard deviation. Superscript equal letters in the same column do not differ statistically by the Tukey test at the 5% level of significance.

Table 2. Behaviour of sugars, glycerol and mannitol in sugarcane molasses wort after treatments

Treatment	Sucrose	Fructose	Glucose	Glycerol	Mannitol	TRS
	g l^{-1}					
Control	102.03 \pm 0.09 ^a	22.47 \pm 0.31 ^b	22.55 \pm 0.14 ^b	<LoQ	0.28 \pm 0,01 ^{ab}	152.15 \pm 0.52 ^a
10 kGy	102.77 \pm 0.47 ^a	21.04 \pm 0.89 ^{bc}	22.24 \pm 1.43 ^b	<LoQ	0.28 \pm 0,01 ^{ab}	151.19 \pm 2.44 ^a
20 kGy	103.30 \pm 3.79 ^a	20.04 \pm 1.00 ^c	20.88 \pm 1.55 ^b	<LoQ	0.30 \pm 0,06 ^a	149.38 \pm 4.33 ^a
40 kGy	99.65 \pm 0.09 ^a	20.35 \pm 0.54 ^c	21.00 \pm 0.65 ^b	<LoQ	0.27 \pm 0,01 ^{ab}	145.99 \pm 1.19 ^a
80 kGy	101.49 \pm 2.30 ^a	20.10 \pm 0.11 ^c	21.05 \pm 0.13 ^b	<LoQ	0.22 \pm 0,02 ^b	147.72 \pm 2.65 ^a
Steam	94.19 \pm 0.27 ^b	26.05 \pm 0.10 ^a	25.08 \pm 0.02 ^a	<LoQ	0.27 \pm 0,00 ^{ab}	150.02 \pm 0.40 ^a
CV	1.82	2.8	4.09	0	9.02	1.58

<LoQ: lower than Limit of Quantification. Averages of $n=5$ \pm standard deviation. Superscript equal letters in the same column do not differ statistically by the Tukey test at the 5% level of significance.

Table 3. Microbiological and biochemical parameters after the fermentation

Treatment	Total bacteria	pH	Glycerol	Mannitol	Sucrose	Glucose	Fructose	Produced during the fermentation				
								Alcohol Content	Yeast biomass	Productivity	Yield	Yeast viability
	Log (CFU ml ⁻¹ +1)							m m ⁻¹	g ml ⁻¹	g l ⁻¹ h ⁻¹	%	%
Control	5.55 ± 0.37 ^a	4.38 ± 0.06 ^{cb}	15.37 ± 0.67 ^a	0.41 ± 0.02 ^a	<LoQ	0.26 ± 0.02 ^a	0.09 ± 0.02 ^a	6.82 ± 0.19 ^c	0.14 ± 0.01 ^a	0.85 ± 0.02 ^{ac}	88 ± 2 ^c	81 ± 2 ^a
10 kGy	2.68 ± 0.40 ^b	4.44 ± 0.11 ^b	15.64 ± 0.25 ^a	0.39 ± 0.01 ^{ab}	<LoQ	0.25 ± 0.07 ^a	0.12 ± 0.02 ^a	7.10 ± 0.13 ^{ab}	0.15 ± 0.00 ^a	0.89 ± 0.02 ^{ab}	92 ± 2 ^b	80 ± 2 ^a
20 kGy	2.25 ± 0.05 ^b	4.27 ± 0.02 ^c	15.83 ± 0.52 ^a	0.37 ± 0.02 ^{ab}	<LoQ	0.48 ± 0.28 ^a	0.14 ± 0.04 ^a	7.09 ± 0.04 ^{ab}	0.15 ± 0.00 ^a	0.89 ± 0.01 ^{ab}	93 ± 1 ^{ab}	79 ± 2 ^a
40 kGy	2.60 ± 0.49 ^b	4.58 ± 0.05 ^a	15.91 ± 0.31 ^a	0.33 ± 0.03 ^b	<LoQ	0.51 ± 0.44 ^a	0.12 ± 0.01 ^a	6.96 ± 0.13 ^{bc}	0.16 ± 0.02 ^a	0.87 ± 0.02 ^{bc}	93 ± 2 ^{ab}	81 ± 2 ^a
Steam	2.20 ± 0.15 ^b	4.65 ± 0.05 ^a	15.65 ± 0.20 ^a	0.35 ± 0.08 ^{ab}	<LoQ	0.28 ± 0.17 ^a	0.09 ± 0.03 ^a	7.28 ± 0.04 ^a	0.14 ± 0.00 ^a	0.91 ± 0.00 ^a	95 ± 0 ^a	82 ± 2 ^a
CV	10.88	1.43	2.74	10.70	0	7.68	22.22	1.73	5.85	1.73	1.72	2.79

CFU: Colony Forming Unit, <LoQ: Lower than Limit of Quantitation, CV: coefficient of variation. Averages of n=5 ± standard deviation. Superscript equal letters in the same column do not differ statistically by the Tukey test at the 5% level of significance.

Table 4. Operating cost relative to the use of electron beam for different doses of radiation applied to sugarcane molasses wort

Treatment	Radiation dose (kGy)	Δt (s)	Cost (US\$)
T0	0	0	0
T1	10	11,6	0,644
T2	20	23,2	0,128
T3	40	46,4	0,258
T4	80	92,8	0,515

Figures

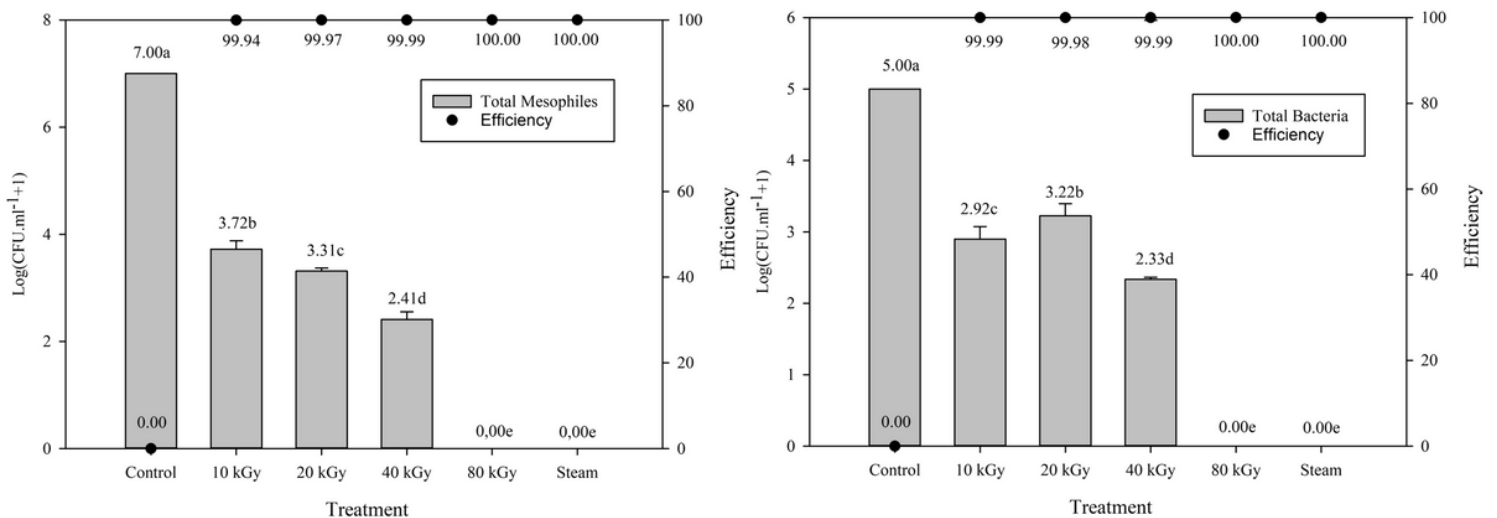


Figure 1

Total mesophiles and total bacteria in sugarcane molasses wort after electron beam irradiation treatment in different doses. The error bars represent the standard deviation.

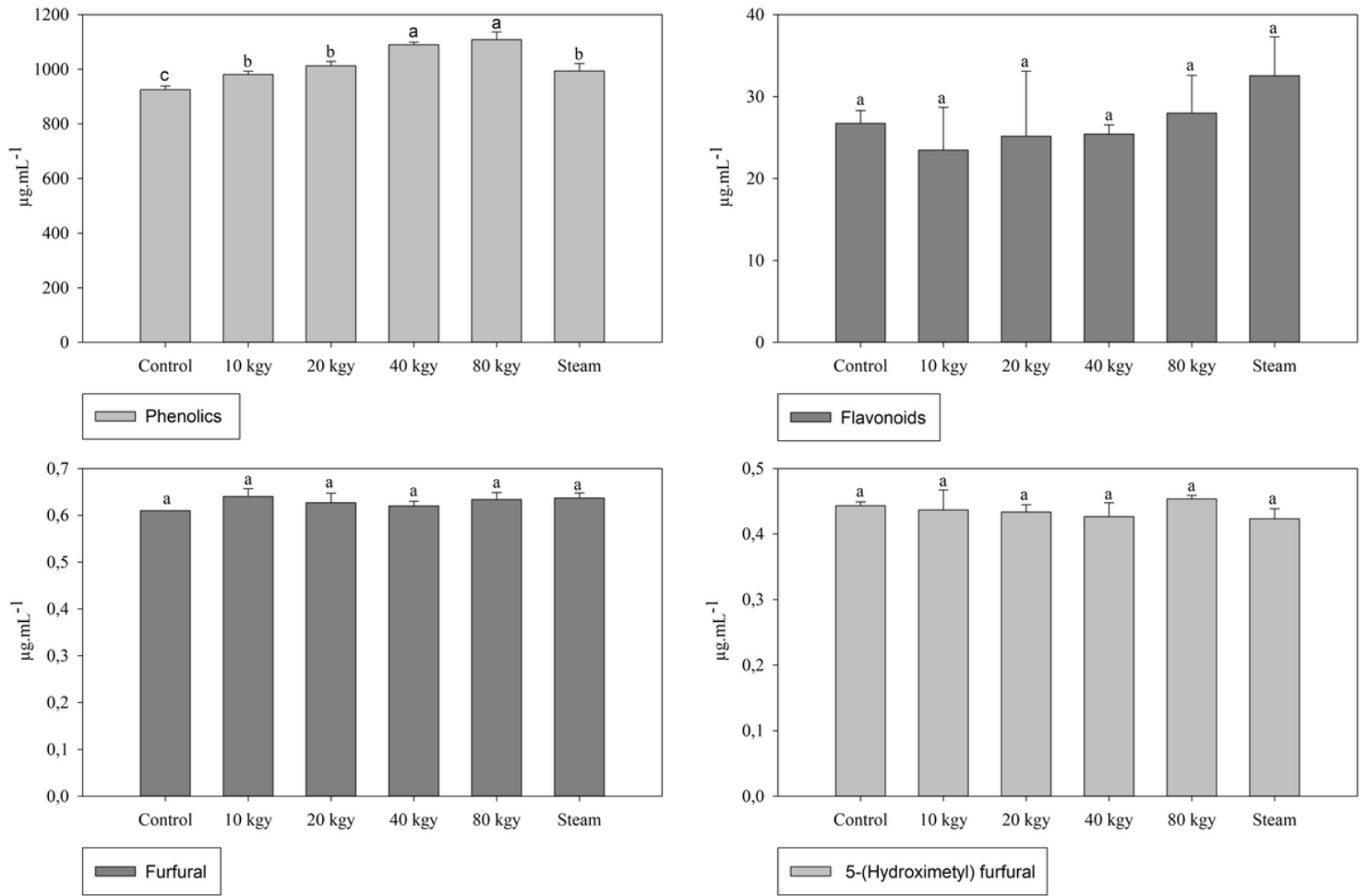


Figure 2

Inhibitors concentrations in sugarcane molasses wort after treatments The error bars represent the standard deviation

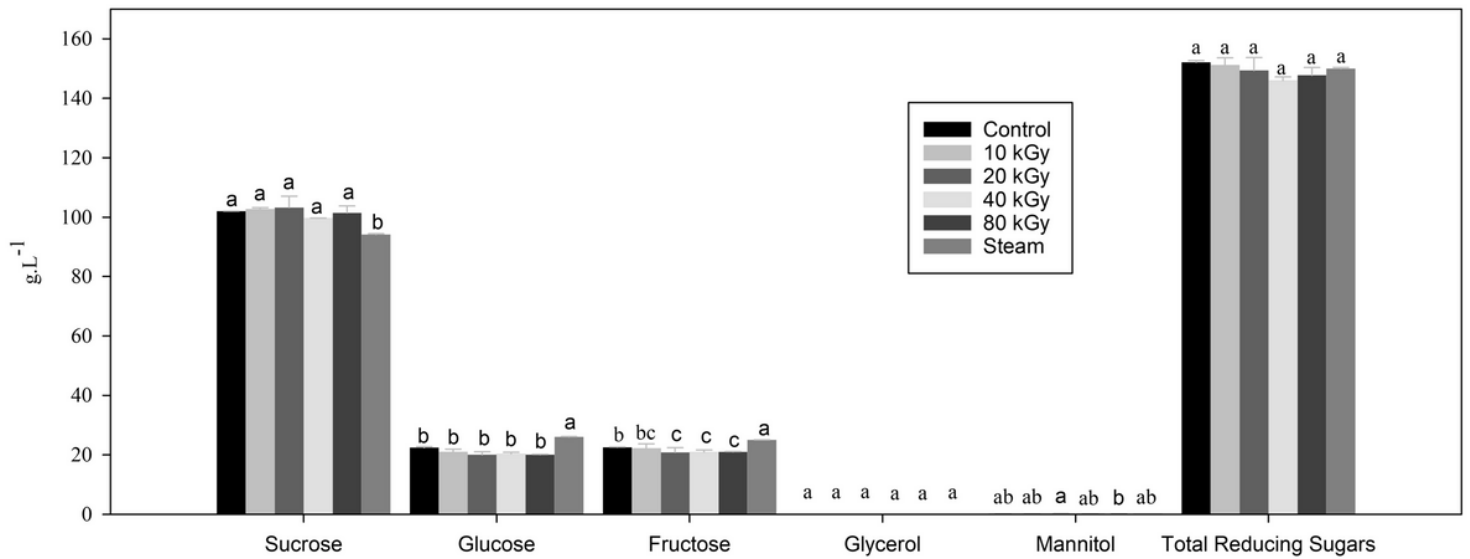


Figure 3

Sugars concentrations at the start of fermentation The error bars represent the standard deviation