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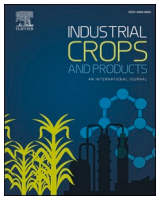

Comparison of sterilization techniques on different feedstock for sugar preservation and bioethanol fermentation

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ABSTRACT

Although bioethanol production is already well-established in several countries, there are stages of the production process that could still be optimized. Feedstock storage is often one of the least efficient part of the bioethanol production process because no sugar preservation techniques are used, which more than often results in sugar waste to microbial contamination. In addition, the trend for future biorefineries is the use of diverse feedstocks, which implies as well that there might be different storage requirements involved. Hence, this study aimed to compare the preservation efficiency of three sterilization techniques on sugar beet molasses and juice including filtration, pasteurization, and ozonation. Results showed that for the molasses, filtration and pasteurization allowed conservation of 80% of the total fermentable sugars, while ozone treatments allowed maintaining 65% readily available for downstream fermentation. In the case of beet juice, pasteurization and ozonation allowed a higher conservation of total fermentable sugars (61% and 41%, respectively) than the filtration treatment for which only 29% was achieved. The sterilization techniques did not have a negative impact on the fermentation performance, with positive controls presenting similar efficiency to fermentation control (~70%). It hence shows that the feedstock composition is a key element in the identification of the most suitable and if possible cost-effective preservation technique.

1. Introduction

Bioethanol is produced by fermenting sugar solutions which are highly prone to microbial contamination prior processing, which can result on up to 80% of sugar loss during the first 15 days (Silva and Sauvageau, 2014; Syed Abdullah et al., 2021). Consequently, biological contamination of the fermentation feedstock could represent an important economic loss, especially in the bioethanol sector where every loss counts (Littlejohns et al., 2018). So far, the most studied and applied preservation technique involves substrate inhibition of microbial growth due to osmotic pressure in solutions with a high specific gravity. Sugar concentrations as high as 600 g/L (Vargas-Ramirez et al., 2016) and 800 g/L (Eggleston et al., 2015) were able to inhibit microbial growth during several months. However, feedstock concentration is a time and energy demanding process (Vargas-Ramirez et al., 2017). Hydrolysates produced from biomass should most probably initially have a lower sugar content than crops such as sugar beet (Vargas-Ramirez et al., 2016) and sweet sorghum (Eggleston et al., 2015). Likewise, the energy required to apply the aforementioned technique on cellulosic hydrolysates is even higher (Saini et al., 2015).

In addition of sugar concentration, a very effective preservation technique that has previously been reported is acidification, which was tested in various sugar sources for fermentation application. For instance, Vargas-Ramirez et al. (2013) reported that a pH lower than 3.5 allowed preserving 99% of fermentable sugars during 24 weeks in sugar beet juice with an initial sugar concentration of 600 g/L. Using a lower sugar concentration (300 g/L) a pH of 3.0 was able to preserve 100% of the fermentable sugars of sugar beet molasses for at least 30 days (de Medeiros Dantas et al., 2022a,2022b). Besides, the use of acid proved to also be efficient for the preservation of solid biomass (Yuan et al., 2022). In the latter, the acid allowed the densification and the hydrolysis of the corn stover while inhibiting microbial growth during 60 days.

However, in bioethanol feedstock preservation, important details that are relevant for food products, like color and flavor, are not as

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relevant for biofuel production (Šopík et al., 2022). Thus, the intuitive way of approaching such situation could imply that the harshest treatments could be the most effective way to avoid microbial contamination. However, this is still not the case once harsh treatments can hinder fermentation and increase production costs. The use of acid has two main drawbacks when it comes to its neutralization. The first one is the obvious yet unavoidable additional cost to the process related to the acidification and neutralization (Vargas-Ramirez et al., 2017), and the second one is due to the increase in the osmotic pressure generated from salt formation. An alternative approach to avoid the use of extremely low pH for long-term storage could be by decreasing the initial microbial load (Dantas et al., 2023).

In light of this, there are different sterilization techniques that proved to be efficient for sugar-rich solutions. Pasteurization is a traditional technique for milk and juice preservation, and when used on sweet sorghum juice, it allowed promoting the sugar conservation for 21 days with a downstream fermentation efficiency of 93% (Kumar et al., 2013). Pasteurization treatment before fermentation was used to ensure the yeast predominance during fermentation of beet molasses (Mikulski and Kłosowski, 2022). Another well-known approach is filtration, which is a easily scalable physical technique that can be used to remove high molecular weight particles like microorganisms (Panigrahi et al., 2018). An ultrafiltration with a 30 kDa membrane successfully improved the preservation of juices by reducing the viable bacterial count in sugarcane juice from 6.63 log CFU/mL to 1.43 log CFU/mL (Panigrahi et al., 2020b). Ozone treatment is another outstanding technique that could be used in sugar solution and enable the fermentation post-storage (Cullen et al., 2010; Dziugan et al., 2016). It has a high cytocide activity and it is already used in wastewater decontamination (Al-Hashimi et al., 2015; Jamil et al., 2017).

From what can be observed in literature, one of the pillars of bioethanol production feasibility is the diversity of feedstocks, considering that the most abundant biomass in the region should be considered for its utilization (De Laporte and Ripplinger, 2019). Nowadays, this reality applies to starch- and sugar-based feedstock (sugarcane, sugar beet, sorghum), but it should also very well apply to waste-based biorefineries in the future (such as cellulosic sugars) (Yaashikaa et al., 2022). Considering an industry that can use different sources for bioethanol production (Garcia-Ochoa et al., 2021), it is important to know which preservation technique could be used or should be adapted to the changing feedstock.

Different studies have been focusing on the optimization of the storage techniques on specific feedstock intended for ethanol production (Klasson and Boone, 2021; Passoth et al., 2009; Vargas-Ramirez et al., 2016; Yuan et al., 2022; Zhang et al., 2018). However, there is so far no comparison on how such methodology is going to behave in different feedstocks. Hence, this study aims at evaluating the impact of different sterilization techniques (filtration, pasteurization and ozonation) on the fermentable sugar preservation and fermentation performance of sugar beet juice (SBJ) and molasses (SBM) at two initial pH (6.0 and 2.0). Although they were obtained from the same crop, the two feedstocks had different composition because one was a primary source, while the other was a waste. This study will therefore be investigating if it can be possible to define a general solution for sugar preservation and how this should be useful in the bioethanol industry.

2. Methodology

2.1. Feedstock characterization

The sugar beet molasses (SBM) and juice (SBJ) were kindly provided by a local sugar refinery in Coaticook (Québec, Canada). Both feedstocks characterized using different physical-chemical methods. First, the soluble solids content was measured using a 3852 PAL-RX/RI refractometer (Atago, Tokyo, Japan). Then, the pH was measured at 20 °C by a pHmeter SYMpHONY (VWR, Canada). The density was measured by gravimetry, using a 25 mL volumetric flask at 20 °C. A viscometer #400, U145 (Cannon-Fenske, USA) was used to measure the viscosity. In a water bath at 30 °C, the time interval for which the liquid meniscus took to successively reach both etched marks was recorded. Then, the dynamic viscosity, η (m^2/s) was calculated using Eq. (1).

$$\eta = viscometer\ constant \times t \times \rho \quad (1)$$

Where ρ is the fluid density (kg/m^3), t (s) is the time that the fluid takes to go from one meniscus to the second meniscus, while the viscometer constant (m/kg) is given by the manufacturer. The moisture and total solid content were quantified by mass loss after drying 5.0 g of each substrate in aluminum weighting dishes at 105 °C during 24 h. The dishes were previous dried at the same temperature before being weighted. The ashes were quantified by calcination at 575 °C for 12 h of the pre-dried samples. All analyses were performed in triplicate.

2.2. Sterilization techniques and storage

The raw SBM and the SBJ were diluted to until 25 °Bx, which resulted in a sugar concentration of 270 g/L and 200 g/L respectively. The final concentration was based on previous preservation studies performed by the group (Dantas et al., 2023). Then, the two feedstocks were subjected to three different methods of sterilization (Fig. 1), when 500 mL of diluted feedstock was used per treatment.

Ozonation was done by the injection of ozone (0.01 g O_3/min) into the respective feedstock during 60 min. The set-up was composed of an oxygen concentrator (AirSep Corporation, USA) and an ozone generator (A2Z Ozone, USA). Pasteurization was done by heating the feedstocks up to 121 °C (15 psi) for 15 min in an autoclave after which they were cooled in an ice bath during 10 min. The filtration was carried out with a membrane Minikros sampler 0.2 μm PES and 20 cm of length (Repligen, USA) coupled in a Quixstand benchtop system (GE Healthcare, USA) and a peristaltic pump using a flowrate of 20 mL/min. After filtration, the membrane was cleaned with backflush of distilled water and stored in a 70% v/v ethanol solution as indicated by the supplier.

After the sterilization processes, the feedstocks had their pH adjusted to 6.0 and 2.0 (Control +) with a H_2SO_4 10 M solution. Samples without treatment were also stored and they are referred as 'Control -' Thus, 60 mL of each different system was transferred to 100 mL glass bottles sealed with rubber stoppers and aluminum rings. After that, they were all stored in accelerated conditions in an incubator at 37 °C (Heratherm incubator, Thermo Scientific, Canada), without agitation, for 42 days. The vials had their internal pressure released every other day and a liquid sample was collected at the beginning and at the end of the storage. Each system consisted in triplicates and the results were expressed as their mean value and standard deviation.

2.3. Analytical procedures

The sugar profile of all samples were determined using a DIONEX ICS-5000 + chromatography system, according to the method previously published (de Medeiros Dantas et al., 2022a,2022b). The organic acids and other possible metabolites were quantified by an Agilent 1100 series HPLC (Agilent Technologies Inc., USA) and the methodology used was previously reported in literature (Beigbeder et al., 2021). For both chromatographic techniques, the samples were diluted in the respective mobile phase and filtered in 0.20 μm individual filters. The total proteins were quantified by the Bradford method, using bovine serum albumin (BSA) (Sigma, USA) as standard (Bradford, 1976).

Total nitrogen was quantified by the Spectroquant® Nitrogen (total) Cell Test Kit (Merk, USA). The nitrate (NO_3^-) and phosphate (PO_4^{3-}) where quantified by the DIONEX ICS-5000 + chromatography system as well. The system was composed by a AS-11HC 4 mm× 4 μm column and a 149 mA suppressor. The flow rate was 1 mL/min and 5 μL of the sample at 10 °C was injected. The eluent used was a KOH gradient and the

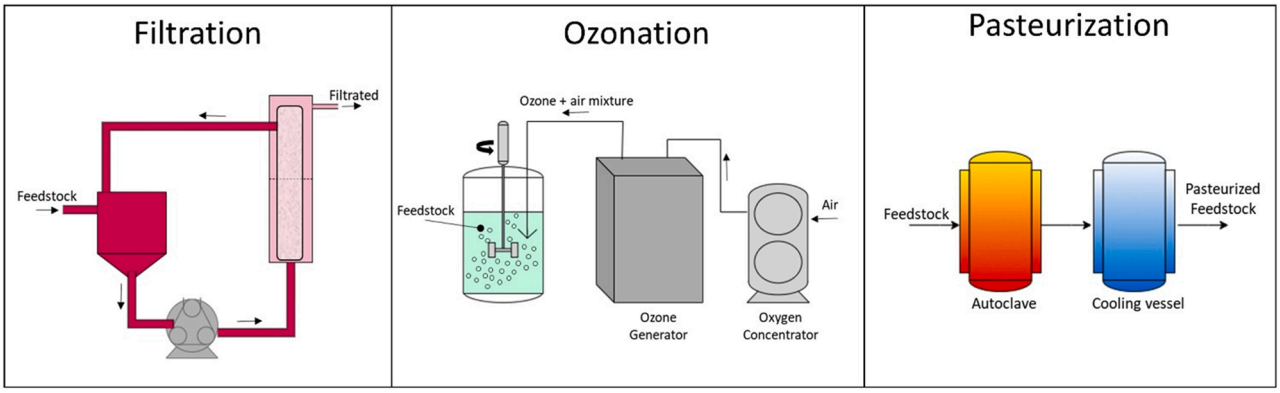

Fig. 1. Schemes of filtration, ozonation and pasteurization experimental systems used to treat SBM and SBJ.

column temperature was 45 °C. The standards used were sodium chloride 99% (Sigma-Aldrich), sodium fluoride 99% (Sigma-Aldrich), sodium bromide > 99% (Alfa Aesar), sodium nitrate 99% (EMD Millipore), sodium sulfate anhydrous (Fisher Scientific) and sodium phosphate monobasic monohydrate (Fisher Scientific).

2.4. Flow cytometry analysis

The samples before and after the sterilization processes were analyzed by flow cytometry to observe the effects on the solids, and possible cells, presented in the feedstocks. As a qualitative analysis of particles in suspension, the samples of SBM and SBJ (before and after treatments) were analyzed using cytometry. The analyses were performed on a BD Accuri C6 flow cytometer (BD Bioscience, Canada). Side (SSC-A) and forward scattering (FSC-A) signals of individual events passing through the laser zone were collected as logarithmic signals. Each sample was diluted 50 times with filtered deionized water before the analysis. Each run lasted 1 min in a flow rate of 150 µL/min. Analyses were performed in triplicates.

2.5. Feedstock fermentation

Fermentation experiments were performed after storage to evaluate which effects the sterilization techniques would have on the final fermentation potential of the feedstocks. For both feedstocks, the replicates of each group were mixed. Sugarbeet molasses and juice (640 g/L in molasses and 690 g/L in juice) that were stored at − 20 °C were added as extra fermentation groups and those were considered 'fermentation control'. The molasses were supplemented with Fermaid-O (Lallemand Biofuels & Distilled Spirits, Canada) at a final concentration of 4.0 g/L. After that, the pH of all media was adjusted to 5.5 using a NH_4OH 10 M solution prior to yeast inoculation. Besides these, any other modification was done to the samples between storage and fermentation.

The inoculation was done with *Saccharomyces cerevisiae* (Thermosacc® DRY, Lallemand Biofuels & Distilled Spirits, Canada). Previously, a solution with yeast and warm tap water was incubated for 15 min at 30 °C, and 140 rpm, in a shaking incubator (Ecotron, Infors-HT Inc., Switzerland). The yeast solution concentration was calculated so that the final dry yeast concentration in the fermentation media would be 1.0 g/L when added as an inoculum of 2.5% (v/v) to the media. The fermentations were carried out in 50 mL serum vials with 30 mL of working volume. After inoculation, the vials were capped with rubber stoppers and aluminum rings. N_2 was flushed inside each bottle for 5 min to ensure anaerobic conditions. Finally, the vials were incubated at 30 °C, 140 rpm, during 96 h (Ecotron, Infors-HT Inc., Switzerland). All the conditions were carried out in triplicate.

2.6. Statistical analysis

One-way analysis of variance (ANOVA) was applied to the experimental data, followed by a Tukey's test. A confidence level of 95% was used ($p\text{-value} \leq 0.05$). Calculations were performed using the software STATISTICA 7.0 (TIBCO Software Inc., Palo alto, CA, USA).

3. Results and discussion

3.1. Initial characterization

Although they both originated from the same crop (sugar beet), juice and molasses are different categories of feedstock. A juice passed through fewer processes (slicing, diffusion and concentration) (Vargas-Ramirez et al., 2017), while a molasses is a byproduct from sugar production (Grahovac et al., 2016). This difference is also highlighted through their different compositions (Table 1). The SBJ has a higher ashes content, which indicates a higher content of minerals as well as having much more proteins, total nitrogen, nitrates and phosphates (Hamouda et al., 2016). It is also important to emphasis that these values will vary according to the harvest, region and processes implemented in the beets. In the case of Vučurović et al. (2018), thick juice presented less ashes (2.0% versus 7.9%) and total nitrogen (1.2% versus 1.8%) than molasses. Therefore, the SBJ is, for the targeted purpose, a richer media than the SBM. The more nutrients a solution has, the more it is prone to microbial growth meaning that by composition, SBJ would be more sensitive to microbial contaminations.

Table 1
Initial physical-chemical characterization of sugar beet juice and molasses before sterilization treatments.

Parameter	Sugar Beet Molasses	Sugar Beet Juice
°Brix (%)	64 ± 0.1	69.8 ± 0.1
pH	6.4	5.8
Density (kg/L)	1.30 ± 0.02	1.30 ± 0.01
Viscosity (Pa•s)	0.10 ± 0.00	0.20 ± 0.00
Moisture (%)	38.77 ± 0.03	34.29 ± 0.62
Total solids (%)	61.23 ± 0.03	65.71 ± 0.62
Ash (%)	1.28 ± 0.01	1.81 ± 0.01
Sugars and organics compounds		
Sucrose (g/L)	563.95 ± 6.34	617.8 ± 40.7
Glucose (g/L)	68.07 ± 1.26	64.80 ± 4.00
Fructose (g/L)	46.78 ± 1.35	56.6 ± 3.2
Lactic acid (g/L)	6.08 ± 0.03	8.51 ± 1.79
Formic acid (g/L)	0.31 ± 0.17	-
Total Proteins (g/L)	2.97 ± 0.20	8.57 ± 0.65
Total Nitrogen (g/L)	1.59	2.51
Anions		
Nitrate (NO_3^-) (g/L)	-	0.79 ± 0.01
Phosphate (PO_4^{3-}) (g/L)	1.64 ± 0.15	2.48 ± 0.02

3.2. Effects of sterilization techniques on feedstock particles

SBJ and SBM were subjected to different sterilization processes to investigate if they would behave in similar ways to each technique. The flow cytometry is used to analyze different characteristics in suspended particles, mainly cells. Hence, the objective of this analysis was to evaluate if these techniques would modify potential microbial cells. However, the feedstocks naturally have many other particles in suspensions such as inorganic particles, vegetable debris and vegetable cells, being difficult to separate microbial cells from those other. Fig. 2 shows the profile of total solid particles before (control groups) and after each sterilization treatment, while the axis FSC-A indicates the particle size, and the SSC-A indicates the particle roughness.

Before any treatment (control samples), the molasses already presents a very different particle profile as compared to the juice. Molasses had much less particles which were mostly similar in size and form, while the juice seems to have a broader variety in particle types and sizes, as well as having overall more of them in the mixture. After the treatments, it could be observed that all preservation techniques had visible effects on both feedstocks and the effects were significantly more intense on the juice than on the molasses for all techniques. For instance, filtration was very efficient for particle removal, as it shows a reduction of those points on the chart when compared to the control. On the other hand, pasteurization modified the particles profile, changing their size and rugosity, which might indicate some potential damage on microbial cells (Schenk et al., 2011). The same effect, but with higher intensity, was observed in the samples subjected to ozonation, especially on the juice, where the rugosity increased drastically. This represents strong evidence that ozonation was effective to damage microbial cells that could potentially occur in the juice.

3.3. Pasteurization effect on sugar preservation

Pasteurization is a well-stablished sterilization technique largely used for sugary solutions by the food industry. Although thermal sterilization techniques can degrade sugars (Bhattacharjee et al., 2019), pasteurization can be used as pretreatment for fermentation feedstocks with negligible alteration on sugar composition (Zhang et al., 2022). Therefore, it was necessary to evaluate if pasteurization was effective to decrease microbial load in the feedstocks. Microbial growth can results in gas production, and the presence of such gas if often an indicative of microbial contamination (Monono et al., 2019). Thus, as the direct quantification of such gases implies on complex set-ups, it can be quantified indirectly by the system weight before and after the gases relief. Only from analyzing the mass loss during storage at 37 ^{o}C, the behavior of treated SBM and SBJ are very similar, presenting a delayed and lower mass loss in comparison with the respective negative controls (Fig. 3 A). The sugar degradation of SBJ, however, was faster, reaching the maximum gas production after just 7 days, while SBM did not reach the maximum mass loss after 42 days (Fig. 3 A). That can be explained by the higher nutrient content and lower initial sugar concentration of SBJ (200 g/L), which constituted a more favorable media for microbial growth than SBM with its 270 g/L of initial sugar.

The sugar profile (Fig. 3B) confirmed the effectiveness of the pasteurization in sugar preservation for both feedstocks, even when not paired with pH 2.0. When analyzing gas production (Fig. 3 A) and sugar profile (Fig. 3B), SBJ suffered a higher sugar degradation than SBM. Pasteurization alone was able to preserve 80% of the sugars from SBM while only 61% of SBJ carbohydrates. Kaavya et al. (2018) analyzed many papers from the literature regarding storage of sugarcane juice after pasteurization and a majority of them runs for less than 45 days at temperatures lower than 10 ^{o}C. Thus, the results from this work are in accordance with what is possible to achieve in terms of sugar preservation using only pasteurization (Kaavya et al., 2018).

The metabolites' increase after storage is a strong evidence of microbial activity (Fig. 3 C). SBJ presented a high increase in the amount of ethanol and lactic acid concentration both for the treated and negative control groups. Lactic acid increased from 2 g/L to 18 g/L, and 14 g/L in the treated and negative control of SBJ. On the ethanol side, the treated SBJ ended with 20 g/L while the negative control reached 60 g/L. Thus, these metabolites can indicate that most of the contamination in the SBJ is done by lactic acid bacteria and yeasts, while in SBM, yeasts seem to be the most prevalent contamination since only an increase in ethanol content was observed. The increase in the organic acids could be noted by the decrease of the pH for both feedstocks by the end of the storage period (Table 2). Hence, the pH measurement is also a good indicative of microbial contamination.

The last important parameter of the sterilization techniques comparison is their impact on the fermentation performance. It is important to highlight that SBM and SBJ were stored with a sugar concentration that enabled direct fermentation (Vučurović et al., 2018). Thus, when the storage ended, the samples were test analyzed for sugar concentration without previous dilution.

The main concern regarding the sterilization techniques is that it could have a negative effect on fermentation after storage. For pasteurization, the differences between the positive control and the fermentation control for fermentation efficiency were not statistically significant (for $p \leq 0.05$) for SBM and SBJ. Thus, it can be concluded that the lower ethanol production in the treated groups was due to the inhibitors generated from microbial contamination, and not as an aftermath of the pasteurization process. Kumar et al. (2013) also evaluated the fermentation yield after storage of pasteurized sweet sorghum juice and did not observed any decrease in yield due to the pasteurization (Kumar et al., 2013).

3.4. Filtration effect on sugar preservation

Filtration was the second sterilization process applied to the feedstocks. For both SBM and SBJ, the treated samples had their sugar degradation delayed (Fig. 4 A), but at the end, the sugar preservation had no statistical difference from the negative controls (Fig. 4B). As it happened with pasteurization, SBM presented less sugar loss than SBJ, once SBM's negative control had a final 68% of sugar preservation while the SBJ's negative control presented just 29% of sugar preserved. Filtration treatment did not promote a high sugar stability in nutrient-rich juices, such as in case of sugarcane juice, where it induced preservation for just 9 weeks at 4 ^{o}C (Panigrahi et al., 2020b).

The metabolites profile (Fig. 4 A) reinforced the other results regarding the microbial contamination. Especially when it came to SBJ, there were no significant difference between the treated sample and the negative control. This shows that the filtration system was not efficient to remove cells/spores present in the juice, although Fig. 2 had shown a visual decrease of the solids by cytometry. Hou et al. (2021) did microbiological characterization in chestnut rose juice filtered with a 100 kDa membrane, and verified that the total aerobic bacteria decreased from 5.13 log CFU/mL to 4.13 log CFU/mL and the chance in yeasts and molds were from 3.15 log CFU/mL to 2.07 log CFU/mL (Hou et al., 2021). This can explain why the filtration retarded the sugar degradation, because it decreased the number of microbial cells, but it did not remove all of them. Besides the high ethanol content generated by the microbial contamination, in both samples, there were the formation of acetic and lactic acid. Thus, some of the sugar was lost to an unwanted product.

The acid production during storage is also reflected by the pH in the treated and negative control samples for SBJ and SBM (Table 3). However, although SBM had the presence of metabolites after storage, their concentration did not compromise the ethanol production, since the ethanol content between the SBM's treated and positive control were statistically the same. On the other hand, SBJ samples barely had any sugar left to ferment to ethanol since the treated sample showed 6.8 g/L of ethanol, while the positive control had 84.3 g/L of final ethanol content.

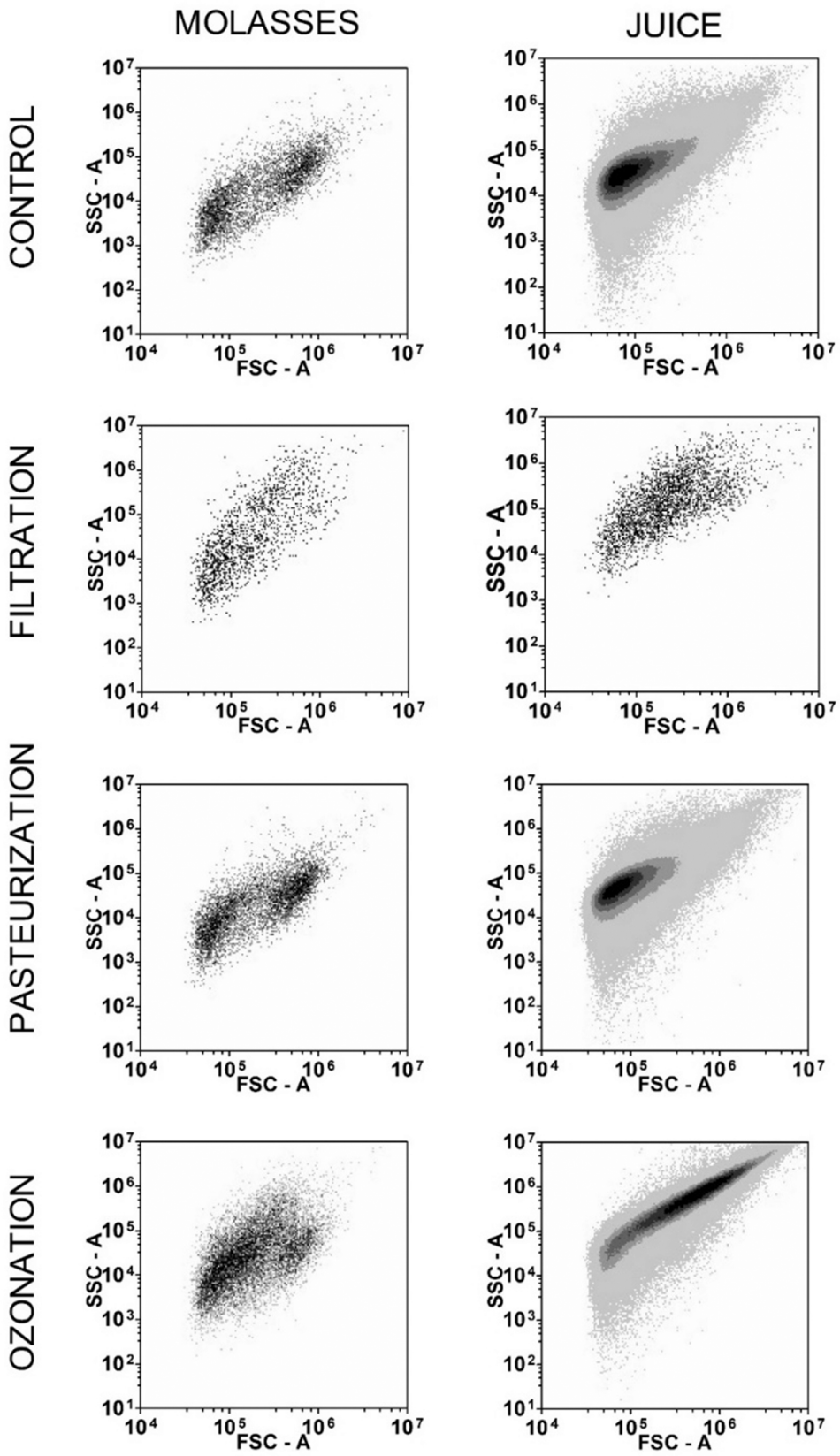

Fig. 2. Particles profiles in sugar beet molasses (left column) and juice (right column) before and after different sterilization techniques (filtration, pasteurization and ozonation). The dot plots represent forward scatter light (FSC) versus side scatter light (SSC), where each dot represents one particle.

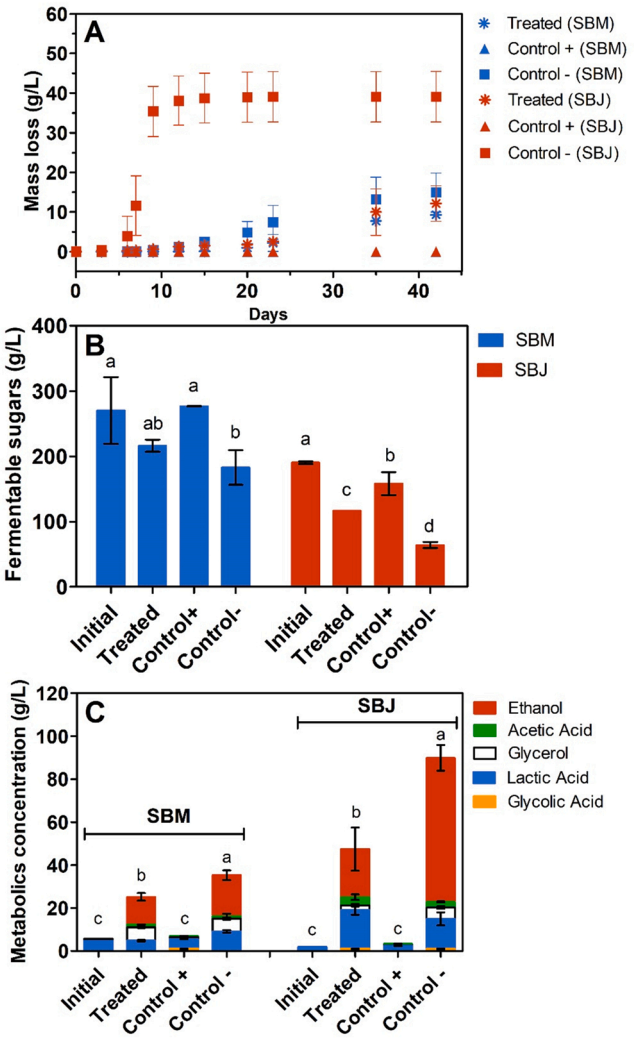

Fig. 3. (A) Mass loss during storage time, (B) sugar profile and (C) metabolites profile at the beginning and end of storage of SBM and SBJ at 37 °C, being 'Treated' the group treated with pasteurization and initial pH of 6.0, 'Control + ' the group treated with pasteurization and initial pH of 2.0 and 'Control -' is the not treated group. Different letters means that the groups were statistically different for a $p \leq 0.05$.

The highlight is that filtration was not harmful at all for fermentation, so when paired with low pH (positive control), it had similar fermentation efficiencies as compared to the fermentation control, namely 67% and 73% for the SBM samples in contrast to 72% and 74% to SBJ. Filtration efficiency can be improved by reducing the pore size of the membrane. The membrane used in this study had a pore size of 0.2 μm, and Zhang et al. (2021) used a of 0.1 μm membrane for removing particles other than pigment and sugars (Zhang et al., 2021). However, an decrease of the pore size implies in increased processing costs due to decrease in the permeate flux or the necessity to use more powerful pumps (Zhang et al., 2021).

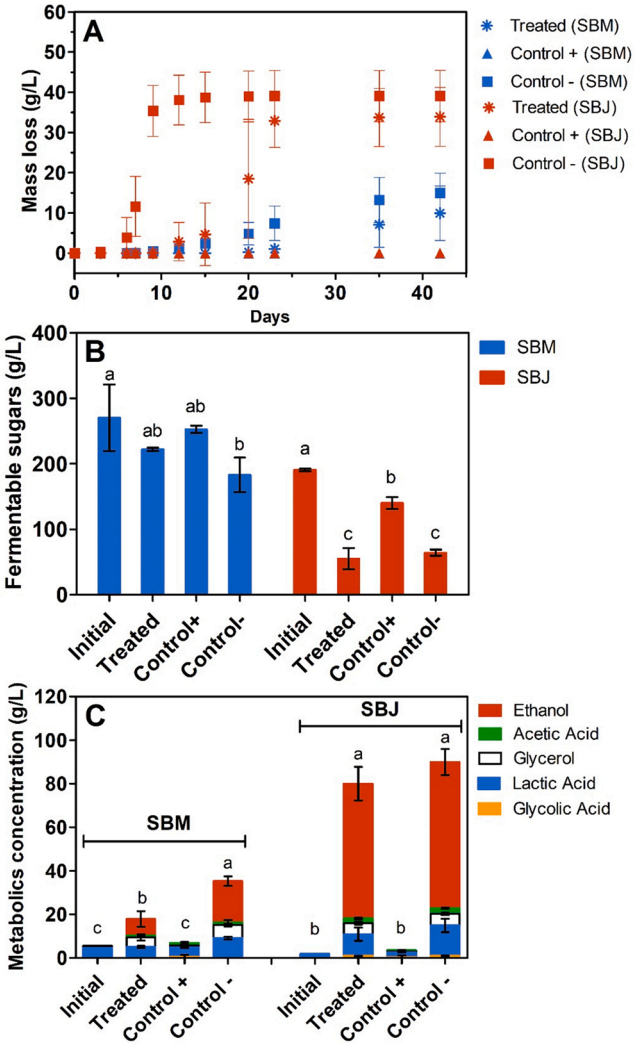

Fig. 4. (A) Mass loss during storage time, (B) sugar profile and (C) metabolites profile at the beginning and end of storage of SBM and SBJ at 37 °C, being 'Treated' the group treated with filtration, 'Control + ' the group treated with filtration and initial pH of 2.0 and 'Control -' is the not treated group. Different letters means that the groups were statistically different for a $p \leq 0.05$.

Table 2
Change in pH before and after storage and bioethanol fermentation performance after storage of groups treated with pasteurization, positive and negative control. Different letters means that the groups were statistically different for a $p \leq 0.05$.

Conditions	Initial pH	Final pH	Ethanol (g/L)	Substrate utilization rate (g/L/h)	Productivity (g/L/h)	Yield Coefficient (g/g)	Fermentation Efficiency (%)
SBM							
Treated	6.0	4.9	78.90 ± 2.37^{b}	1.52 ± 0.34^{a}	0.84 ± 0.03^{b}	0.60 ± 0.15^{a}	58.14 ± 2.36^{a}
Control +	2.0	1.9	98.91 ± 1.58^{a}	2.54 ± 0.12^{a}	1.05 ± 0.02^{a}	0.42 ± 0.03^{a}	68.32 ± 2.88^{a}
Control -	6.0	4.2	56.92 ± 4.68^{c}	0.78 ± 0.33^{b}	0.65 ± 0.08^{c}	1.02 ± 0.44^{a}	45.57 ± 7.32^{b}
Fermentation control	-	-	92.5 ± 9.14^{a}	2.00 ± 0.09^{a}	0.93 ± 0.07^{a}	0.47 ± 0.01^{a}	72.90 ± 3.48^{a}
SBJ							
Treated	6.0	3.7	22.58 ± 15.78^{b}	1.60 ± 0.35^{b}	0.22 ± 0.20^{b}	0.12 ± 0.10^{b}	22.98 ± 2.06^{b}
Control +	2.0	2.0	67.21 ± 7.16^{a}	2.85 ± 0.02^{a}	1.06 ± 0.08^{a}	0.37 ± 0.02^{a}	73.06 ± 5.88^{a}
Control -	6.0	4.1	0.56 ± 0.79^{b}	0.38 ± 0.36^{c}	0.01 ± 0.01^{b}	0.07 ± 0.10^{b}	1.80 ± 2.55^{c}
Fermentation control	-	-	68.86 ± 2.03^{a}	1.96 ± 0.02^{b}	0.73 ± 0.02^{a}	0.40 ± 0.02^{a}	73.57 ± 2.89^{a}

Table 3
Change in pH before and after storage and bioethanol fermentation performance after storage of groups treated with filtration, positive and negative control. Different letters means that the groups were statistically different for a $p \leq 0.05$.

Conditions	Initial pH	Final pH	Ethanol (g/L)	Substrate utilization rate (g/L/h)	Productivity (g/L/h)	Yield Coefficient (g/g)	Fermentation Efficiency (%)
SBM							
Treated	6.0	4.9	82.31 ± 6.06^{b}	1.01 ± 0.71^{b}	0.88 ± 0.06^{b}	1.42 ± 0.79^{a}	49.80 ± 6.06^{b}
Control +	2.0	1.9	90.68 ± 13.62^{b}	2.54 ± 0.15^{a}	0.96 ± 0.14^{b}	0.38 ± 0.05^{a}	66.82 ± 9.51^{a}
Control -	6.0	4.2	56.92 ± 4.68^{c}	0.78 ± 0.33^{b}	0.65 ± 0.08^{c}	1.02 ± 0.44^{a}	45.57 ± 7.32^{b}
Fermentation control	-	-	92.5 ± 9.14^{a}	2.00 ± 0.09^{a}	0.93 ± 0.07^{a}	0.47 ± 0.01^{a}	72.90 ± 3.48^{a}
SBJ							
Treated	6.0	3.7	6.80 ± 6.92^{b}	1.08 ± 0.71^{c}	0.07 ± 0.07^{b}	0.10 ± 0.15^{b}	20.02 ± 28.35^{c}
Control +	2.0	2.0	84.29 ± 9.69^{a}	2.47 ± 0.15^{a}	0.90 ± 0.10^{a}	0.37 ± 0.03^{a}	71.54 ± 6.27^{b}
Control -	6.0	4.1	0.56 ± 0.79^{b}	0.38 ± 0.36^{c}	0.01 ± 0.01^{b}	0.07 ± 0.10^{b}	1.80 ± 2.55^{c}
Fermentation control	-	-	68.86 ± 2.03^{a}	1.96 ± 0.02^{b}	0.73 ± 0.02^{a}	0.40 ± 0.02^{a}	73.57 ± 2.89^{a}

3.5. Ozonation effect on sugar preservation

Ozone is a strong oxidation agent and it is known to target microorganisms by inactivating them (Jamil et al., 2017; Panigrahi et al., 2020a). It is usually used for wastewater treatment (Nahim-Granados et al., 2020) although here it was used to sterilize the sugar-rich feedstocks. As for the two other techniques, its efficiency in sugar preservation was compared with untreated samples (negative control) and with samples treated with a combination of ozone and acid (positive controls).

Different from the other two techniques afore discussed, the ozonated samples did not produced many gas during storage (Fig. 5A). However, in this specific case, this fact did not reflect on a low sugar loss. As Garud et al. pointed out in their research, the increase rate of aerobic mesophilic bacteria in sugarcane juice was the same for the control and juice treated just with ozone (Garud et al., 2018). As can be seen in Fig. 5B, the SBM treated sample and the negative control sugar content had no significant difference, with a final preservation of 65% (treated sample) and 68% (negative control). Also, considering the positive control, it presented a certain sugar loss, presenting 90% of sugar preservation at the end of the storage. This is important to be highlighted since it is the lowest preservation percentage for SBM positive control between the three techniques studied in this work.

When considering the SBJ, which has proven to be a more microbial contamination-sensitive feedstock, the ozonation seems to be even less effective. The technique does have a positive effect on decreasing the microbial contamination, comparing the treated sample with 41% of sugar preservation and the negative control with 34% although the positive control just presented a 63% sugar preservation. However, when analyzing the metabolites profile (Fig. 5 C), there is no formation of metabolites in the positive control when compared to the initial profile. Thus, the sugar could have been degraded through the continuous oxidation of the ozone, since the oxygen radicals would not be selective on with molecules they would target. Marcq et al. (2009) studied the kinetics of sugar oxidation by ozone and could quantify the production of different species, such as gluconic acid and glycolic acid (Marcq et al., 2009).

The lack of metabolites formation in the SBJ positive control is confirmed by the pH (Table 4) since it remained at 2.0. This reinforces the hypothesis that the ozone oxidized the sugar and transformed it into other compounds. Now, when analyzing the fermentation results, one can conclude that for the SBM, the ozonation did not produced hindering effect to the fermentation, besides the negative control, all efficiencies were statistically the same. In the case of SBJ, the differences were drastic because of the higher sugar loss, however the ethanol production of the positive control was not affected by the sugar loss when compared with fermentation control.

Therefore, although the ozone degraded some of the sugar, the process did not produce fermentation inhibitors, as it can be seen when comparing fermentation efficiencies of positive controls and

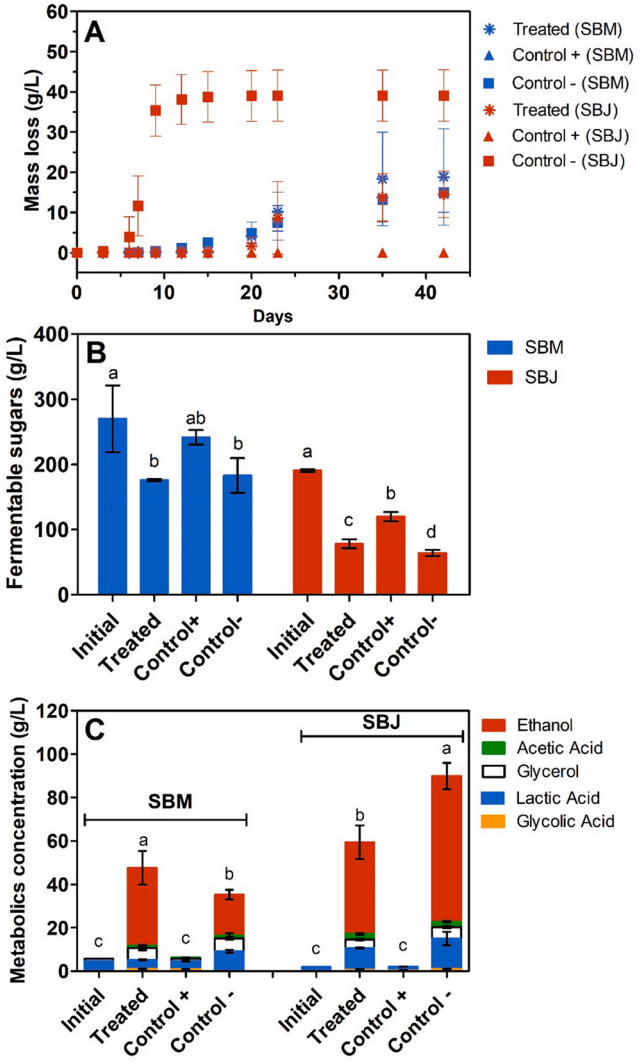

Fig. 5. (A) Mass loss during storage time, (B) sugar profile and (C) metabolites profile at the beginning and end of storage of SBM and SBJ at 37 °C, being 'Treated' the group treated with ozonation, 'Control + ' the group treated with ozonation and initial pH of 2.0 and 'Control -' is the not treated group. Different letters means that the groups were statistically different for a $p \leq 0.05$.

fermentation controls of SBJ and SBM. The results were in agreement with what Dziugan et al. observed with the fermentation of sugar beet juice treated with 15 min of ozonation, thus no difference with the performance of fresh raw juice and the ozonated juice (Dziugan et al., 2016). The main reason for any side effects of ozone on the fermentation

Table 4
Change in pH before and after storage and bioethanol fermentation performance after storage of groups treated with ozonation, positive and negative control. Different letters means that the groups were statistically different for a $p \leq 0.05$.

Conditions	Initial pH	Final pH	Ethanol (g/L)	Substrate utilization rate (g/L/h)	Productivity (g/L/h)	Yield Coefficient (g/g)	Fermentation Efficiency (%)
SBM							
Treated	6.0	4.9	45.22 ± 6.98^{c}	0.62 ± 0.41^{b}	0.39 ± 0.09^{c}	0.75 ± 0.35^{a}	66.81 ± 9.45^{a}
Control +	2.0	1.9	72.86 ± 12.65^{b}	2.17 ± 0.09^{a}	0.81 ± 0.16^{b}	0.38 ± 0.09^{a}	69.77 ± 16.38^{a}
Control -	6.0	4.2	56.92 ± 7.32^{c}	0.78 ± 0.33^{b}	0.65 ± 0.08^{c}	1.02 ± 0.44^{a}	45.57 ± 7.32^{b}
Fermentation control	-	-	92.5 ± 9.14^{a}	2.00 ± 0.09^{a}	0.93 ± 0.07^{a}	0.47 ± 0.01^{a}	72.90 ± 3.48^{a}
SBJ							
Treated	6.0	3.7	20.47 ± 7.87^{b}	1.09 ± 0.38^{c}	0.22 ± 0.08^{b}	0.24 ± 0.16^{b}	46.01 ± 29.88^{b}
Control +	2.0	2.0	74.85 ± 8.55^{a}	2.26 ± 0.08^{a}	0.80 ± 0.09^{a}	0.36 ± 0.06^{b}	68.60 ± 10.83^{b}
Control -	6.0	4.1	0.56 ± 0.79^{b}	0.38 ± 0.36^{c}	0.01 ± 0.01^{b}	0.07 ± 0.10^{b}	1.80 ± 2.55^{c}
Fermentation control	-	-	68.86 ± 2.03^{a}	1.96 ± 0.02^{b}	0.73 ± 0.02^{a}	0.40 ± 0.02^{a}	73.57 ± 2.89^{a}

is because it has a short life-time in water (around 20 min), thus it did not hindered the yeast metabolism during fermentation (Rosen et al., 2022).

4. Conclusions

Our study provides valuable insights on how the selection of a storage technique has an impact on fermentable sugar preservation and on the downstream ethanol production. Although originating from the same crop (sugar beet), the juice and molasses were generated from different processes, resulting in two distinct compositions. Since the three sterilization techniques investigated in the work resulted in unique effects for each feedstock, the idea of defining a one-size-fits-all storage system for maximize ethanol production for a wide range of feedstock seems unrealistic. Therefore, the search for sugar preservation treatments that can be standardized for different types of feedstocks should be pursued.

The combination of pH 2.0 with a sterilization technique allowed preserving sugar beet molasses and juice for 42 days in accelerated conditions. So, when there is not an exorbitant difference between the techniques, a techno-economic, life-cycle and scale-up analyses becomes necessary to assess in the decision-making process of which technique is the most suitable for specific substrate. Therefore, this work is intended to stimulate discussion on how the reality of sugar preservation will be carried out in biorefineries that will be fed with heterogeneous feedstocks.

CRediT authorship contribution statement

Julia Maria de Medeiros Dantas: Conceptualization, Methodology, Investigation, Writing – original draft. **Javier Ricardo Gómez Cardozo:** Supervision, Writing – review & editing. **Jean-Baptiste Beigbeder:** Conceptualization, Writing – review & editing. **Jean-Michel Lavoie:** Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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