Biomass and Bioenergy 169 (2023) 106655

Contents lists available at ScienceDirect

Biomass and Bioenergy

journal homepage: www.elsevier.com/locate/biombioe

Combination of different preservation techniques as low-cost strategies inhibiting sugar degradation in liquid feedstock used for bioethanol fermentation

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ARTICLE INFO

Keywords:
Sugar beet molasses
Fermentation
Bioethanol
Ozone
Oil layer

ABSTRACT

Just like for the food supply chain, sugar-based feedstock used in the bioethanol industry might be affected by biological contamination, resulting in important economic losses. Therefore, it is necessary to develop cost-effective storage techniques capable of preserving feedstock over several months. In this study, three different techniques (ozonation, acidification, and addition of a corn oil layer) were investigated individually as well as combined for the long-term storage of sugars dedicated to fermentation. After 300 days of storage, up to 100% of the initial fermentable sugars were preserved with acidification (pH 2.0), independently or in conjunction with other conservation techniques. However, in terms of fermentation potential, the groups treated with acid had a lower ethanol yield than the groups maintained at pH 6.0. When the solution was treated with ozone combined with acidification, it yielded 55.71% of bioethanol efficiency, whereas when treated only with ozonation at pH 6.0, fermentation reached a 67.20% yield. Ozonation was found to be efficient to preserve fermentable sugars, either with the use of an oil layer (80% conservation) or at lower pH (100% ethanol efficiency). Finally, the combination of ozonation and an oil layer presented the highest total ethanol production, reaching 101g L^{-1} after 96h of incubation using *Saccharomyces cerevisiae*. Thus, the combined use of ozonation and an oil layer has the potential to be used in the bioethanol sector as a system to preserve liquid feedstock without the use of evaporation to increase sugar concentration.

1. Introduction

Although biofuels are considered an alternative to fossil fuels, the traditional way to produce bioethanol still involves considerable amounts of carbon [1]. A carbon-efficient production process relies on low energy-intensive processes [2] combined with the use of energy-efficient feedstock such as agro-industrial by-products and energy crops [3]. Bioethanol can contribute to reducing GHG emissions because the use of a gasoline-ethanol mixture involving 30% EtOH can reduce GHG emissions by 7.2% when corn is used as feedstock, and by up to 13.4% when cellulose is used as feedstock [4]. However, feedstock that is generally used for bioethanol production can be subjected to microbial contamination from the moment of harvest up to industrial processing, thus potentially causing economic loss [5]. Sweet sorghum, for example, can lose 49% of its fermentable sugars in one (1) week [6] while for sugar beets, the maximum storage time is 6 weeks in north-eastern Colorado, a temperate-climate region [7]. As an example of microbial contamination, several microorganisms such as *Salmonella* spp. [8], *Enterobacter* spp. [9] or *Aspergillus* spp. [10] were detected in natural sugarcane and in sugar beet juices.

A traditional method to avoid microbial contamination of the feedstock is by drying it, since water is an essential element for microbial growth [11]. Such an approach is often recommended for low water-content crops such as corn. However, drying is not feasible for crops with high water content (*e.g.*, sugar beet, sugarcane, and sweet sorghum). For this type of feedstock, preservation of the extracted juices has several advantages over conservation techniques that would involve the whole crop, including a decrease of storage volume requirements, a decrease in transportation costs [12], and a longer preservation time [13]. Techniques used in the food sector are good examples of the

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https://doi.org/10.1016/j.biombioe.2022.106655
Received 30 March 2022; Received in revised form 24 October 2022; Accepted 13 November 2022
Available online 30 December 2022

effectiveness of preserving sugar-rich liquids [14]. In addition, the chosen preservation technique should not have high associated costs while enabling yeast fermentation after storage to improve profitability in bioethanol production [15]. Moreover, in the specific case of fuel ethanol production and in opposition to the methodologies used for food products, there is no concern about changing the organoleptic aspects of the sugar mixtures.

Some of the methods reported in literature are classified as sterilization procedures since they aim at decreasing or destroying the initial microbial load [16]. The use of high temperature, such as for pasteurization, is a common approach used in the food sector [17]. The sterilization thermal process mechanism includes membrane damage as well as protein, and DNA denaturation [18]. Nonetheless, the disadvantage of thermal treatments in the case of carbohydrate solutions is that high temperatures also lead to sugar degradation, such as in the Maillard reaction for example, and inhibitor formation [19].

Ozonation has been reported as an efficient sterilization method to remove microorganisms such as *Escherichia coli* and *Salmonella* spp. [20] as well as for the treatment and disinfection of wastewater [21]. Garud et al. (2018) observed a significant effect of ozone on aerobic mesophilic bacteria, with a decrease from 6.1 log cells to 2.2 log cells after 10 min of ozone treatment [22]. Thus, ozonation can be considered a suitable technique for sterilizing liquid feedstock for bioethanol production because it does not involve heat and can be easily scaled up using a bubble column [23].

Although sterilization methods are effective in decreasing the microbial load, they do not promote any mechanism to act against new contaminants during storage. The addition of chemical preservatives could modify the media composition, making it unsuitable for microbial growth during storage [17]. For wines for example, sulfites are used for preservation due to their antimicrobial activities [24]. Similarly, sodium metabisulphite and acetic acid are used as preservatives in coconut water, while aiding in preventing rancidity and allowing control over discoloration [25]. Likewise, the use of organic acids, *e.g.* citric and benzoic acid, is effective for the preservation of cashew apple juice [26], while sodium benzoate along with sorbic acid has been reported efficient for the preservation of sweet sorghum juice [27].

The majority of microorganisms are not able to grow at extreme pH, such as below 4.0 [28] and above 10.0 [29]. Thus, the use of acids is an efficient chemical preservation method for biofuel feedstock because pH is crucial to control cellular processes and ultimately, low pH can be adjusted by neutralization before fermentation [30]. For applications in the food sector, the type of acid used can be important since it can affect characteristics such as flavor and color. Nonetheless, for fermentation feedstock, Monono et al. [31], reported that there is no significant difference in terms of sugar preservation by using different types of acid as long as the pH is lower than 4.0.

In addition to pH changes, the reduction of water activity is another alternative to avoid microbial growth in sugar-rich solutions. The availability of water for cellular processes as well as an isotonic environment with the cellular interior is important to allow microbial growth [32]. Indeed, the concentration of solutions with sugar concentrations between 60°Bx [29] and 80°Bx [13] was shown effective for the long-term storage of feedstock intended for the production of ethanol. However, water evaporation requires intense energy use, which is not favorable to the carbon intensity of the whole process [33].

The addition of a vegetable oil layer on top of the liquid feedstock has been highlighted as a low energy and easy method to be implemented for the preservation of fermentable sugars. Klasson et al. (2021) showed that the oil layer contributed positively in hindering microbial growth at the lower sugar concentrations tested (30°Bx) without affecting fermentation after storage [34]. The use of two or more preservation techniques in the same media has been shown more effective than a single method when it came to sweet sorghum juice (chemical and evaporation) [35], sugarcane juice with ozone and lactic acid [22], and sugarcane juice with a mix of natural preservatives [36]. Thus, the storage of sugar feedstock (intended to produce bioethanol) with such a low sugar content ($\leq$250 g L^{-1} of sugars) for more than 180 days has never been reported in literature. The longer the sugar preservation, the better it would be for industrial purposes because it means that industrial plants will be able to operate all year long, and not just during crop season (as they do right now for corn ethanol as an example). Consequently, this would involve smaller plants to process the same amount of feedstock, since operating time will be longer, decreasing operational and capital costs as well.

Considering all the aforementioned points, it seems crucial to develop a preservation methodology that requires less energy for long-term conservation of liquid feedstock used in fermentation processes, while avoiding the loss of carbon during bioethanol production. Storage conditions (room temperature and low sugar concentration) are otherwise extremely favorable to microbial growth. Therefore, by combining three different preservation techniques, such as ozonation, acidification, and an oil layer, this study aims to propose a methodology capable of preserving mildly concentrated sugar solutions (220 g L^{-1}) in the long term. Furthermore, it was evaluated whether the combination of techniques allowed the preservation of fermentable sugars while still producing high ethanol yields after storage.

2. Materials and method

2.1. Sugar beet molasses

This study is aimed at mimicking a complex sugar solution that could be used as feedstock for bioethanol production. To this purpose, sugar beet molasses (initial concentration of 655 g L^{-1} of fermentable sugars) were diluted with distilled water to a concentration of 250 g L^{-1} of fermentable sugars. The dilution objective was to decrease the sugar concentration of the solution to evaluate how the preservation techniques would behave in lower sugar concentrations. The molasses was kindly provided by a local sugar refinery located in Coaticook (Québec, Canada). Table 1 presents the physical-chemical characteristics of the molasses before dilution.

2.2. Preservation methodology

This study aims to evaluate the combination of three different preservation techniques (sterilization, chemical, and physical) for the long-term storage of fermentable sugars. The techniques were previously screened individually by our team [37]. First, the 100 mL glass bottles that were used to contain the system were washed with soap and thoroughly rinsed with distilled water. After being air dried, they were autoclaved for 30 min at 121°C. For media ozonation, the set-up was composed of an oxygen concentrator (AirSep Corporation, USA), and an ozone generator (A2Z Ozone, USA) was used to inject ozone into the sugar solution for 60 min. The ozone generator was operated at a 50% concentration of O_3 in an oxygen stream and at a flowrate of 0.01

Table 1
Physical-chemical characteristics of sugar beet molasses produced by a semi-industrial sugar refinery.

Parameter	Value
Density (kg·L^{-1})	1.29
Brix (°Bx)	64
Sucrose (g·L^{-1})	560.95
Glucose (g·L^{-1})	55.23
Fructose (g·L^{-1})	39.00
Lactic acid (g·L^{-1})	11.28
Acetic Acid (g·L^{-1})	0.79
Viscosity (Pa·s)	0.10
TOC (g·L^{-1})	371.00
Total solids (%)	64.70
Ashes (%)	1.23
pH	6.36

g_{O3}·min^{-1}. The chemical treatment was performed by acidifying the sugar solution to pH 2.0 with 10 M H_2SO_4 (7.1 mL H_2SO_4·L^{-1} sugar solution). Finally, corn oil was used as a physical layer to protect the media from external contamination. A 1.5 cm layer of oil was disposed over the 60 mL of media. The volume of corn oil should vary depending on the geometry of the recipient, since the target was on layer thickness. Therefore, the groups presented a combination of the different levels (Table 2) of each treatment, in 8 different systems.

After treatment, the bottles were sealed with rubber stoppers and aluminum rings. Storage was then performed for 300 days at room temperature (20 ± 2 °C) in the dark. The vials had their internal pressure released every 10 days and a liquid sample was collected every 60 days. A syringe was used to collect the sample through the rubber stoppers. Syringes and needles were rinsed with water and cleaned with a 70% v·v^{-1} ethanol solution between each sampling. Each system was replicated three times and the results expressed here represent an average value to which standard deviation was associated.

2.3. Fermentation experiments

One of the most important features of the preservation systems was to avoid hindering ethanol production after storage time. Hence, fermentations were performed after the storage assay. If present, the oil layer was removed, after which the three replicates of each group were mixed together. Then, the fermentation media were supplemented with 4 g L^{-1} of Fermaid-O nutrient (Lallemand Biofuels & Distilled Spirits, Canada) and the pH was adjusted to 5.5 using NH_4OH, 28% (Anachemia, Canada) prior to yeast inoculation.

The inoculum was prepared using *Saccharomyces cerevisiae* (Thermosacc® DRY, Lallemand Biofuels & Distilled Spirits, Canada), which first involved re-hydrating the dry yeast cells in warm tap water during 15 min at 30°C in a shaking incubator (Ecotron, Infors-HT Inc., Switzerland) at 140 rpm. The inoculum solution was transferred to 30 mL of fermentation media in a 2.5% (v·v^{-1}) proportion using 50 mL serum vials in order to reach an initial yeast concentration of 1.0 g L^{-1} in the fermentation media. The fermentation bottles were capped with rubber stoppers and aluminum rings after they were flushed with N_2 for 5 min to ensure anaerobic conditions. All fermentation systems were incubated at 30°C and 140 rpm during 90 h. All fermentation assays were carried out in triplicate.

2.4. Cost estimation

The prices considered in this study were gathered in 2021 and the operation costs were estimated from lab (considering 1 L of solution) and pilot (considering 1 m^3 of solution) scales. The estimation was realized considering just the operational costs, such as energy use and chemical supplies. No capital or installation costs were considered in this preliminary evaluation. All chemicals prices were estimated based on what was obtained by the chemical supplier Lab Alley (www.laballey.com, accessed in October 2022). The power cost was the one applied in Québec, CA, in October 2022, for industries of 0.035 CAD/kWh (www.hydroquebec.com, accessed in October 2022). Therefore, the storage cost (C_s) was calculated using equation (1).

$$C_s = C_A + C_{Oil} + C_O \quad (1)$$

Table 2
Lower and upper levels of preservation techniques (ozonation, acidification, and oil layer) applied to the sugar solution (250 g L^{-1}) before storage. Each system had a unique combination of this method, totalizing 8 systems.

Treatments	Lower Level	Upper Level
Ozonation time	0 min	60 min
Acidification	pH not adjusted (pH 6.0)	Adjusted to pH 2.0
Oil layer	No	Yes

Where C_A is the cost of acidification and neutralization to the respective pH, C_{Oil} is the price for the correspondent oil volume used, and C_O is the ozonation cost according to the respective ozonation time.

2.5. Statistical analysis

The statistical analysis was carried out using STATISTICA 7.0 software (TIBCO Software Inc., Palo Alto, CA, USA). One-way analysis of variance (ANOVA) followed by Tukey's test were performed on the experimental data to evaluate and compare the groups individually, using 95% confidence level (*p-value* ≤ 0.05). The effects of each factor are expressed in Pareto charts, using a confidence level of 95% (see Supplemental material).

3. Analytical procedures

3.1. Sugar profile

The sugar matrix used during the study (molasses) was a complex media that presented different sugars, mainly sucrose, glucose, and fructose. A Dionex ICS-5000+ chromatography system was used to determine the sugar profile of the samples qualitatively and quantitatively. The system was composed of a Dionex CarboPac Sa10-4μM column at 45°C, a downstream electrochemical detector operated at 30°C, an analytical gradient pump, and a thermostated AS-AP auto-sampler. In addition, a KOH eluent generator was used to provide a proper eluent concentration, and to ensure signal stability, a 200 mM KOH post-injection system with a Dionex GP 50 gradient pump was implemented. The injection volume was 0.4 μL and aqueous solutions of KOH were used for elution at the following concentrations: 1 mM for 12 min, 10 mM for 5 min, and 1 mM for 1 min at a 1.25 mL min^{-1} flow rate. The calibration curve ranged from 10 ppm to 1000 ppm and the following standards were used: fructose (99%), glucose (99%), and sucrose (99%), which were all purchased from Sigma-Aldrich.

3.2. Metabolites analysis

The presence of organic acids and alcohols are important indicators of microbial metabolism. Thus, their concentrations were evaluated throughout this study. An Agilent 1100 series HPLC (Agilent Technologies Inc., USA) was used, coupled with a G1362A Refractive Index Detector to quantify some of these metabolites. The system involved a G1322A Degasser, a G1311A Quartenary Pump and a G1313A Autosampler while the detector was operated at 40°C. The ROA-Organic Acid H+ (8%) column used for this work was operated at 65°C, and the elution was performed at a constant flow of 0.08 mL min^{-1} using a final 2.5 mM H_2SO_4 concentration. The calibration curve ranged from 10 ppm to 1000 ppm and involved the following standards: formic acid 100% (Fisher Scientific), acetic acid 99.9% (Sigma-Aldrich), glycerol 99% (Sigma-Aldrich), glycolic acid 99% (Sigma-Aldrich), ethanol 99% (Sigma-Aldrich), furfural 99% (Aldrich) and 5-hydroxymethyl furfural 99% (Aldrich).

3.3. Microbial gas production

The production of various gases (*i.e.,* CO_2, CH_4, H_2S) is another factor that indicates microbial growth and metabolite reactions [31]. Considering this, the preservation assessments were carried out in bottles locked with rubber stoppers and aluminum rings, which would allow the gas produced due to an eventual microbial growth to increase the bottles' internal pressure. In light of this, the bottles had their internal gases released every 10 days. The procedure consisted in weighing the bottles, releasing the build-up pressure by inserting a needle through the rubber septum, and then weighing them again. All the production of gases were expressed as g_{gas}·$L^{-1}_{solution}$.

3.4. Carbon mass balance

A carbon mass balance is imperative for the comparison of the preservation performances of the evaluated strategies. Along with the quantification of sugar and metabolites, the balance of total organic and inorganic carbon concentrations provides a better overall view of changes in the carbon profile. The liquid samples at day 0 and 300 were filtered on a 0.45 μm filter (Chromspec, Canada) before being analyzed by a total organic carbon analyzer (Shimadzu TOC-Ve, Japan). The combustion of the samples was performed at 680°C using a platinum catalyst with a flow rate of purified air (150 mL min^{-1}).

The procedure allowed the creation of a detailed carbon balance that can be used as a tool to compare the initial and final carbon content present in the solution. In the quantification of carbon loss by gas release, it was assumed that all gas produced during the conservation assays were CO_2 or CH_4. In some cases, undetected carbon metabolites were observed by the difference between organic carbon contents and the sum of the different carbon compounds quantified during the study (sugar, organic acids, glycerol, biogas, etc.). These undetected organic compounds were referred to as 'non-identified carbon' and discussed in the text.

3.5. Fermentation performances

After storage, the media that were processed using the different treatments were submitted to fermentation to evaluate the effect of storage strategies on fermentation performances. Fermentation kinetics were followed using the mass loss generated due to CO_2 production. Considering the theoretical fermentation reaction: $C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$, 1.0 g of glucose should be converted into 0.51 g of ethanol and 0.49 g of CO_2. Therefore, an estimated ethanol production can be indirectly predicted by the cumulative mass of CO_2 generated by the yeast. The vials were weighed before and after the internal gas release along the fermentation time, and the mass loss was considered being a loss of CO_2 solely. This methodology has been adapted from Beigbeder *et al.* (2021) [38].

This method allowed an instant evaluation of the fermentation kinetics to estimate the end of the fermentation experiment. The final ethanol and glycerol concentrations were quantified using a HPLC (as described in section 3.2). The fermentation responses evaluated were yield coefficient ($Y_{P/S}$) ($g{\cdot}g^{-1}$), productivity rate ($g{\cdot}L^{-1}{\cdot}h^{-1}$), sugar consumption rate ($g{\cdot}L^{-1}{\cdot}h^{-1}$), and ethanol production efficiency (%), as can be seen in the following equations.

$$Y_{P\cdot S^{-1}} = \frac{P_{real}}{S_0 - S_f} \tag{2}$$

$$\text{Productivity} = \frac{P_{real}}{t} \tag{3}$$

$$\text{Sugar consumption rate} = \frac{S_0 - S_f}{t} \tag{4}$$

$$\text{Ethanol production efficiency} = 100 \times \frac{P_{real}}{P_{max}} \tag{5}$$

Where P_{real} ($g{\cdot}L^{-1}$) is final ethanol content, S_0 ($g{\cdot}L^{-1}$) is initial sugar concentration, S_f ($g{\cdot}L^{-1}$) is final sugar content, t (h) is total fermentation time and P_{max} ($g{\cdot}L^{-1}$) is ethanol content if all the fermentable sugars were converted. Considering this, 1 g of glucose or fructose would ideally produce 0.51 g of ethanol.

4. Results and discussion

4.1. Sugar preservation

The storage of feedstocks for ethanol production is a problem inherent to the logistics of biofuel production [39]. Sugar losses during storage time directly affect ethanol yields, which in turn could have been used for energy [40]. Therefore, acid pH, ozonation, and oil layers were combined in 8 different combinations and used as preservation methodology to avoid degradation of fermentable sugars in this study (Table 2). The experimental results are divided between groups with and without oil, in order to allow a simpler overview of the data.

Since gas production is indicative of microbial contamination, mass loss due to pressure release was followed during storage time (Fig. 1). It was observed that, regardless of the presence of oil, the lack of chemical agent (ozonation and acidification) resulted in high gas production for the first 30 days, after which gas production stabilized during the remainder of the storage time. In this situation, the oil had a positive effect since it presented less gas production, which is probably due to the lack of oxygen in the media, turning the environment unfavorable to aerobic species. In addition, the initial microbial contamination might have produced metabolites, which started to reduce progressive growth, as gas production ceased for the majority of the time. The use of ozone and a pH of 2 inhibited gas production in the systems, indicating that it might have an effect against microbial proliferation.

Although gas production is a common indication of microbial contamination, the sugars can be consumed throughout other metabolic routes, thus it was necessary to quantify the sugar content in the systems (Fig. 2). The sugar loss followed the same tendency as the mass loss, with the untreated group losing 40% of fermentable sugars after 60 days and almost 80% after 300 days of storage. It confirms that when any other technique is applied, the oil layer does help withsugar preservation. However, when considering the sugar preservation aspect, the presence of oil was not statistically significant (see Fig. S2 in Supplemental material). When the ozone treatment alone was applied, the presence of oil seemed to boost microbial growth and sugar degradation. However, when no oil was added to the mixture, 100% of the initial sugar were preserved when performing an ozonation pretreatment of 60 min, with no significant differences observed whether the original pH was 6 or 2 ($p\text{-}value \leq 0.05$). The group oil/60 min/pH 2 had a 100% sugar preservation, whereas the group oil/60 min/pH 6 presented degradation of sugar content (80% of sugar preservation). Therefore, when using oil, the sole application of ozone was not sufficient to control microbial growth. In fact, the interaction of ozone and pH is more significant than the single effect of ozonation (see Fig. S2 in supplemental material). During storage, it could be observed that the first indication of microbial contamination was sucrose hydrolysis. However, the acidification to pH 2.0 also promoted hydrolysis of sucrose after 60 days (data not shown), even though total sugar concentration was stable until the end of 300 days (Fig. 2B). For example, it can be predicted that the group no oil/60 min/pH 6 shows microbial contamination, because all sucrose was hydrolyzed, and pH dropped below 6.0 (Table 3).

By taking these results into consideration, some points about the microbial profile present in the liquid feedstock could be discussed although no microbiologic analysis was performed to identify them as previously mentioned. According to Dziugan *et al.*, ozone presents a high sterilization capacity, capable of decreasing the microbial load of microorganism such as *Candida vini* and *Aspergillus brasiliensis* from 10^5 to 0 CFU mL^{-1}, after 30 min of treatment [10]. Ozone acts by disintegrating the cell walls of microorganisms, by lipid peroxidation of the lipopolysaccharide content in the membrane [41].

Under aerobic conditions (without oil layer), the results presented in our study agree with those reported by Dziugan *et al.* (2016), where the ozone treatment was effective to decrease initial contamination of strains and spores whereas fermentable sugars remained unaltered. In addition, it might have produced radicals and other compounds that promoted a preservation system, allowing sugar preservation during the total 300 days of storage. However, the combination of ozone and oil at pH 6.0 led to a degradation of sugar after only 60 days of storage (Fig. 2). This might indicate that, for certain spores of anaerobic strains, 60 min of ozonation might not be sufficient to induce complete destruction,

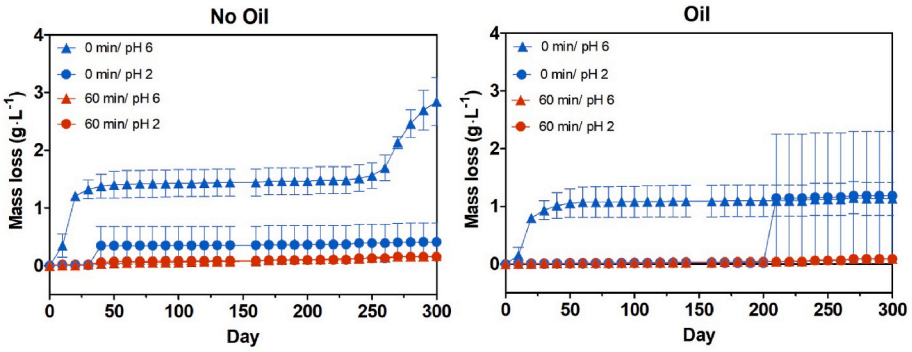

Fig. 1. Mass loss of sugar solution originally at 250 g·L^{-1}of initial sugar during 300 days of storage at room temperature. A combination of oil layer, acid and ozone treatment were applied to the groups, thus involving 8 different treatments. Red lines stand for groups treated with ozonation, blue lines stand for groups not treated with ozonation while triangles stand for tests at pH 6.0 and circles for pH 2.0. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

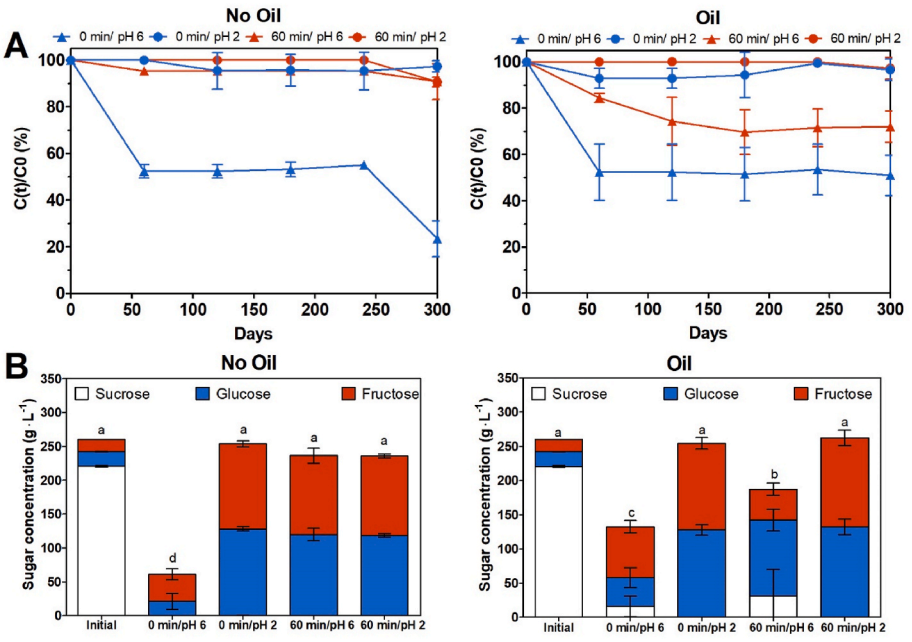

Fig. 2. Fermentable sugar loss (A) and fermentable sugar profiles (B) before and after 300 days of storage at room temperature using diluted sugar beet molasses. A combination of oil layer, acid and ozone treatments were applied to the groups at different intensities. Red lines represent groups treated with ozonation, blue lines represent groups not treated with ozonation, triangles represent pH 6 and circles for pH 2.0. Different letters represent significantly different values for *p-value* ≤0.05. Tukey's test was carried out comparing all the groups, and although they are divided between 'No Oil' and 'Oil', the comparison takes in consideration all eight (8) groups. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Final pH and metabolites from sugar degradation of sugar solution at 250 g·L^{-1}of fermentable sugars stored during 300 days. The one-way ANOVA compares all the groups individually for metabolites. In the case of pH, Tukey's test compares groups with the same initial pH. Different letters represent significantly different values for *p-value* ≤ 0.05.

	Final pH	Glycolic Acid (g·L^{-1})	Lactic Acid (g·L^{-1})	Glycerol (g·L^{-1})	Acetic Acid (g·L^{-1})	Ethanol (g·L^{-1})
Initial solution	6.36	0.0 ± 0.00^{d}	2.19 ± 0.02^{d}	0.11 ± 0.06^{e}	0.0 ± 0.00^{g}	0.00 ± 0.00^{b}
No oil/0 min/pH 6	3.98 ± 0.01^{b}	0.0 ± 0.00^{d}	9.86 ± 0.07^{b}	2.89 ± 0.10^{a}	3.19 ± 0.35^{b}	33.68 ± 8.91^{a}
No oil/0 min/pH 2	2.21 ± 0.02^{a}	0.0 ± 0.00^{d}	4.16 ± 0.34^{c}	1.03 ± 0.02^{cd}	0.50 ± 0.17^{efg}	0.00 ± 0.00^{b}
No oil/60 min/pH 6	4.80 ± 0.02^{a}	1.13 ± 0.04^{a}	3.98 ± 0.12^{c}	1.20 ± 0.09^{bc}	1.41 ± 0.26^{c}	0.00 ± 0.00^{b}
No oil/60 min/pH 2	2.12 ± 0.01^{b}	1.10 ± 0.04^{ab}	4.01 ± 0.11^{c}	1.23 ± 0.00^{b}	0.94 ± 0.14^{cde}	0.00 ± 0.00^{b}
Oil/0 min/pH 6	3.90 ± 0.02^{c}	0.0 ± 0.00^{d}	11.22 ± 0.31^{a}	1.04 ± 0.03^{cd}	3.95 ± 0.04^{a}	0.14 ± 0.19^{b}
Oil/0 min/pH 2	2.22 ± 0.02^{a}	0.0 ± 0.00^{d}	4.31 ± 0.18^{c}	0.94 ± 0.10^{d}	0.37 ± 0.05^{fg}	0.59 ± 0.13^{b}
Oil/60 min/pH 6	4.80 ± 0.01^{a}	1.05 ± 0.01^{bc}	3.97 ± 0.12^{c}	1.25 ± 0.02^{b}	1.22 ± 0.04^{cd}	0.14 ± 0.20^{b}
Oil/60 min/pH 2	2.15 ± 0.01^{b}	1.0 ± 0.04^{c}	3.84 ± 0.09^{c}	1.11 ± 0.06^{bcd}	0.75 ± 0.20^{def}	0.00 ± 0.00^{b}

considering that spores are more resistant to ozone action than vegetative cells [42,43].

In general, the results reported in this work show a significant improvement when compared to the current literature in the field. The use of a "diluted" sugar-based feedstock (250 g·L^{-1}of fermentable sugars) is lower than the concentration used in other published studies which mentioned concentrations such as 600 g L^{-1} [29], 800 g L^{-1} [13] while being even lower than the 300, 400, and 500 g·L^{-1}studied by Klasson *et al.* [34]. Furthermore, the preservation method proposed in our study was shown to be efficient for longer periods, reaching 300 days, while the other studies were carried out for 70 days [34], 160 days [13], and 168 days [29].

4.2. Metabolites and carbon balance

During storage time, a change in the profile of other components in the sugar solutions was also noticed, but more specifically, an increase in the concentration of organic acids (*i.e.*, lactic, glycolic, and acetic acid),

glycerol, and ethanol was observed in some groups. Glycolic acid was only present in the groups that were treated with ozone, which can be explained by the oxidative impact of O_3 on sucrose, thus producing glycolic acid (see supplemental material) [44]. However, glycolic acid levels remained stable during the whole storage time (data not shown).

The presence of metabolites varied according to the preservation method (Table 3). As an example, the group treated with oil only produced higher contents of lactic acid and acetic acid when compared to groups without any treatment. However, the presence of oil promoted higher sugar preservation. This suggests that in the anaerobic environment, the microbiota was more conditioned to lactic and acetic fermentation pathways than other metabolic reactions. For all the other groups, these acid contents were higher than the initial concentration, but still very low ($<$5.0 g L^{-1}). The untreated control group produced a high amount of ethanol, which might indicate the development of microorganisms such as yeast, as the genera *Candida* spp. is commonly found in sugar-rich solutions, such as sugar cane and beet molasses [45].

The pH and metabolites (organic acids, ethanol, furans) at the starting and ending point of storage are shown in Table 3. Regarding pH, groups with pH 2.0 presented a pH variation of $\leq$0.2, which, although it was statistically different when analyzing the system, did not represent a substantial difference. A slight increase in pH values was also reported by Vargas-Ramirez *et al.,* (2013) as they were measuring and readjusting pH along the storage period [29]. When analyzing the groups with pH 6.0, combined with ozonation, the presence of oil had no impact on pH change, since both presented a final pH of 4.8. On the other side, for the groups at pH 6.0 and no ozonation, the oil presence resulted in a lower pH (3.90) than the group with no oil (3.98).

In order to summarize the sugar and metabolites profiles of the groups, an organic carbon balance was calculated to evaluate how the carbon from fermentable sugars was transformed during storage. Fig. 3 shows the distribution of carbon between sugars, metabolites, and gas. The non-identified carbon refers to the excess organic carbon that did not fit into any of the above-mentioned categories.

Fig. 3 shows that the use of acid pH is the most effective among the three techniques considered in this work for sugar preservation, which is confirmed by the Pareto chart (Fig. S2 in supplemental material). Even alone, acid treatment at pH 2.0 presented a high sugar preservation capacity. It is also interesting to notice that in the groups with low sugar preservation, such as Oil/0 min/pH 6, Oil/60 min/pH 6, No Oil/60 min/pH 6, the increase in metabolites was not proportional to the carbon loss from the sugars, however the organic carbon was still in solution. This is another indication that reinforces the microbial contamination hypothesis because this means that the carbon shifted from sugars to other substances that our analytical methods could not identify. Some examples of what those substances can be are amino acids, polysaccharides such as dextrans, and other primary and secondary metabolites, like other organic acids [46].

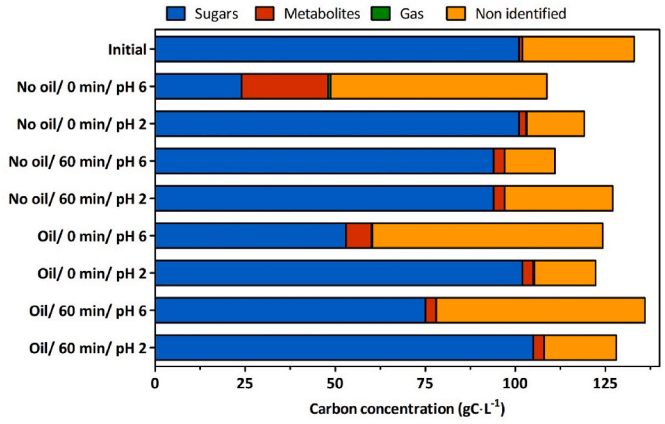

Fig. 3. Carbon distribution of initial sugar solution and samples after 300 days under different storage conditions. The non-identified carbon is defined as the surplus organic carbon after the sugars, metabolites, and gas quantification.

4.3. Fermentation performance

The preservation techniques tested were able to promote high sugar preservation percentage along the 300 days of storage. However, the referred techniques could have a negative effect on the fermentation performance after storage. In light of that, the different groups were submitted to ethanol fermentation after the storage period, as can be seen in Fig. 4.

Even if the oil layer was removed before fermentation, the groups stored with oil had an overall higher production of ethanol than the group without oil. Although the addition of oil was not significant for sugar preservation, it had a positive effect on fermentation yield (see Fig. S3 in supplemental material). Oil has been used before as an accessory technique for sugar preservation, however its beneficial effect on fermentation was not reported before [13,34].

Klasson *et al.* (2021) observed that concentrations of 20 g L^{-1} of lactic acid completely inhibited ethanol fermentation after storage of sugar solution at 30°Bx [34]. In our study, despite the increase in lactic and acetic acid during storage, it did not seem to inhibit yeast fermentation. On the other hand, the use of pH 2.0 negatively affected fermentation (Fig. S3, supplemental material). Although the groups with those lower pH values demonstrated the highest percentages of sugar preservation, they produced as much gas during the fermentation as the other groups, which can be an indication that they produced the same ethanol content. This may be associated with the osmotic pressure caused by the high amount of salt formed during pH neutralization to be suitable for fermentation [47,48].

Fermentation results (Table 4) were in accordance with the kinetics behavior shown in Fig. 4. The measurement by gas loss presented slightly higher results than the analytical results, but this situation has already been reported in literature and may be associated with water being entrained by the gases [38]. Despite this, the fermentation results were positive. All conditions with a high percentage of sugar preservation also generated high ethanol production. According to Darvishi and Moghaddami (2019), 11% (v·v^{-1}) of bioethanol after fermentation is an acceptable content for industrial scale, which they achieved with an optimization of parameters using sugar beet molasses as substrate [49]. Two conditions of our study were able to improve this ethanol content without any fermentation optimization, with 11.38% (No oil/0 min/pH 2) and 12.86% (Oil/60 min/pH 6).

The solution treated with Oil/60 min/pH 6 presented the highest fermentation efficiency. These results are very interesting since, although this conservation condition did not offer 100% of the original sugars found in the mixture, it produced more overall ethanol (101 g L^{-1}) than the conditions that had the best sugar preservation (the groups that were at pH 2). When comparing Oil/60 min/pH 6 and Oil/0 min/pH 2, their produced ethanol content is not statistically different. However, Oil/60 min/pH 6 has a superior efficiency because all the sugar from this group was used, while in the Oil/0 min/pH 2 there was still some sugar left at the end of fermentation.

A hypothesis that might explain the latter is the lower content of salt due to pH neutralization in the media in the groups with pH 6.0 than the groups with pH 2.0. The fact that acidification will present lower fermentation yields was stated by our previous work, where 300 g L^{-1} of sugars were 100% preserved during 30 days by a pH of 3.0 [37]. However, when fermenting this solution, it presented a 50% yield, while the fresh fermentation control led to a 70% of yield.

Also, the slightly lower concentration of sugar in the Oil/60 min/pH 6 group decreased the osmotic pressure, which probably had an impact on yeast growth and secondary metabolites [50]; a strong hypothesis on why such groups could reach the highest yields. When working with highly concentrated sugar media, the discussion of what was more

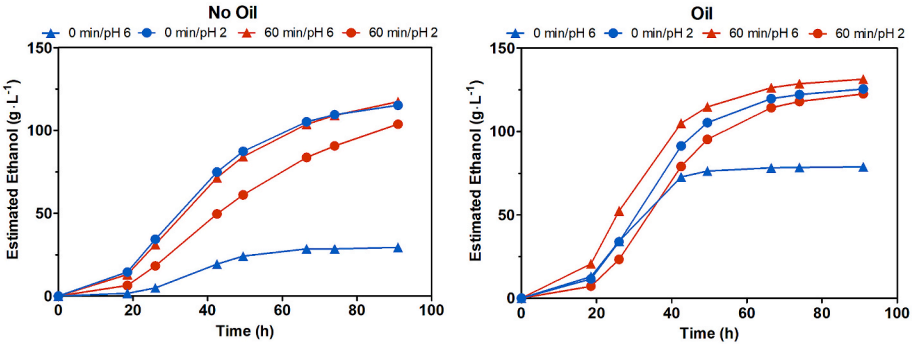

Fig. 4. Fermentation kinetics of sugar solutions after 300 days of storage at room temperatures. Fermentation was carried out at 30°C and 140 rpm. Each group was submitted to different storage conditions and fermentation was carried out in triplicates. Red lines represent groups treated with ozonation, blue lines represent groups not treated with ozonation, triangles represent pH 6 and circles stands for pH 2.0. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 4
Ethanol production responses after fermentation of sugar beet molasses solution under different preservation methodologies after 300 days of storage at room temperature. The one-way ANOVA compares all the groups individually for fermentation parameters. Different letters represent significantly different values for *p-value* ≤ 0.05.

Conditions	Ethanol ($g.L^{-1}$)	Productivity ($g{\cdot}L^{-1}{\cdot}h^{-1}$)	Ethanol % ($v{\cdot}v^{-1}$)	$Y_{P/S}$, Yield coeff. ($g{\cdot}g^{-1}$)	Sugar consumption rate ($g{\cdot}L^{-1}{\cdot}h^{-1}$)	Ethanol production efficiency (%)
No oil/0 min/pH 6	11.05 ± 8.29^{d}	0.12 ± 0.09^{d}	$1.40 \pm 1.05d$	0.15 ± 0.11^{c}	0.67 ± 0.18^{d}	29.77 ± 21.96^{c}
No oil/0 min/pH 2	89.59 ± 12.48^{ab}	0.98 ± 0.14^{ab}	11.36 ± 1.58^{ab}	0.36 ± 0.03^{ab}	2.75 ± 0.15^{a}	68.79 ± 6.34^{ab}
No oil/60 min/pH 6	80.13 ± 5.42^{ab}	0.88 ± 0.06^{ab}	10.16 ± 0.69^{ab}	0.37 ± 0.05^{ab}	2.38 ± 0.16^{ab}	67.20 ± 9.60^{ab}
No oil/60 min/pH 2	66.93 ± 1.78^{b}	0.74 ± 0.02^{b}	8.48 ± 0.23^{b}	0.35 ± 0.01^{ab}	2.07 ± 0.04^{b}	55.71 ± 1.16^{b}
Oil/0 min/pH 6	42.54 ± 3.10^{c}	0.47 ± 0.03^{c}	5.39 ± 0.39^{c}	0.33 ± 0.06^{bc}	1.45 ± 0.20^{c}	64.55 ± 12.63^{b}
Oil/0 min/pH 2	74.30 ± 2.93^{ab}	0.82 ± 0.03^{ab}	9.42 ± 0.37^{ab}	0.30 ± 0.01^{bc}	2.70 ± 0.05^{a}	57.47 ± 2.35^{b}
Oil/60 min/pH 6	101.47 ± 18.67^{a}	1.12 ± 0.20^{a}	12.86 ± 2.37^{a}	0.55 ± 0.07^{a}	2.01 ± 0.16^{b}	100 ± 0.03^{a}
Oil/60 min/pH 2	77.11 ± 4.67^{ab}	0.85 ± 0.05^{ab}	9.77 ± 0.59^{ab}	0.30 ± 0.03^{bc}	2.81 ± 0.19^{a}	57.97 ± 6.14^{b}

harmful to the yeast between osmotic pressure and high ethanol concentration always arises, but in this case, one can observe that the ethanol concentration was not the limiting factor because the yeast was able to increase ethanol production [38]. A production of more than 100 g L^{-1} of ethanol was also achieved in our previous work with a fresh fermentation control of 300 g L^{-1} [37]. Therefore, the use of ozone with the oil layer stands out as the best storage technique for sugar preservation used in bioethanol production.

4.4. Cost estimation of preservation systems

The implementation of sugar preservation techniques from lab to industrial scales must take into consideration the different operational costs such as electricity, heat, and consumables in order to be economically feasible. In light of this, a preliminary cost analysis was performed to evaluate the treatment cost of each individual preservation method as well as their combinations.

Table 5 presents the costs in USD per liter and per cubic meter of sugar solution. For acidification, as mentioned before, H_2SO_4 and NH_4OH were used. In addition, it is important to highlight that acidification is the most expensive treatment when compared to ozonation and oil layer. The ozonation cost at lab scale was calculated according to the power used by our equipment at lab scale and the power required by a pilot scale equipment was considered the one calculated by Acién *et al.* (2012).

After calculating the operation cost of each preservation technique, the costs were combined according to the techniques presented in each system. Table 6 presents the implementation price for each system. The conjoint analysis of cost, sugar preservation, and ethanol yields are essential to decide which preservation method is the best to improve economic feasibility of bioethanol production. As previously discussed, the use of pH 2.0 was very efficient to preserve sugars (more than 85% of sugar preservation independently of any accessory method), but it hindered fermentation, with a correspondent fermentation yield lower than 75%. On the other hand, ozonation is the cheapest technique, since it is mostly electric energy-dependent, and is low energy intensive. Therefore, considering the low implementation cost and the highest ethanol production (101 g L^{-1}), the Oil/60 min/pH 6 system can be considered as the best option for fermentable sugar preservation between the herein tested conditions.

Table 5
Application costs of acidification, ozonation and oil layer at lab scale and pilot scale.

Technique	Level	Lab Scale ($USD{\cdot}L^{-1}$)	Pilot Scale ($USD{\cdot}m^{-3}$)
Acidification + Neutralization	6	0.00	1.00
	2	0.23	226.00
Ozonation	0 min	0.00	0.00
	60 min	0.05	0.22
Oil Layer	0 mL	0.00	0.00
	25 mL	0.08	78.62

Table 6
Lab scale and pilot scale cost for implementation of preservation technique for each tested groups.

Conditions	Lab Scale ($USD{\cdot}L^{-1}$)	Pilot Scale ($USD{\cdot}m^{-3}$)
No oil/0 min/pH 6	0.00	1.17
No oil/0 min/pH 2	0.23	225.83
No oil/60 min/pH 6	0.05	1.39
No oil/60 min/pH 2	0.27	226.05
Oil/0 min/pH 6	0.08	79.79
Oil/0 min/pH 2	0.30	304.45
Oil/60 min/pH 6	0.13	80.01
Oil/60 min/pH 2	0.35	304.68

5. Conclusion

In this work, the storage of low-concentration sugar solutions for bioethanol production was shown to be possible and feasible. A solution containing 250 g L^{-1} of fermentable sugar (diluted sugar beet molasses) was successfully persevered for 300 days under different storage conditions. Such storage conditions (sugar concentration and storage period) have never been reported in literature before, to the best of our knowledge. Acidification to pH 2.0 or an ozone pre-treatment allowed conserving 100% of the initial sugar after 300 days of experiment. However, the combination of both techniques was shown to be too harsh to allow a satisfactory fermentation performance.

However, as far as the ethanol production is concerned, the most severe treatment is not always the best choice. In this case, a slight sugar loss for samples at pH 6.0 with ozonation and oil was shown to be more beneficial for the overall ethanol production as compared to a conservation that would allow recovering most of the sugars although it generated a lower fermentation efficiency. The economic analysis supports this conclusion, since acidification is the most expensive of the three techniques. From this work, it can be deduced that the best storage methodology would be ozonation combined with an oil layer. This study shows some advances in terms of feedstock storage to support the growth and development of a more sustainable and feasible bioethanol production. A future optimization of the ozonation process and oil layer could possibly lead to an improvement of this preservation system.

Data availability

Data will be made available on request.

Acknowledgements

The authors are grateful to the Biomass Technology Laboratory (BTL) at Université de Sherbrooke and especially its sponsors: The Ministère de l'Énergie et des Ressources Naturelles du Québec (MERNQ), NSERC (RDCPJ 531896–18), CRIBIQ (2018-003-PR-C37) without forgetting our industrial sponsor Greenfield Global for funding this work. In addition, the authors would like to acknowledge a local sugar refinery in Coaticook (Québec, Canada) for providing the sugarbeet molasses. Finally, further acknowledgements go to the Biomass, Bioproducts and Bioprocess Analysis Laboratory (L.A.B) for its valuable support regarding various samples analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biombioe.2022.106655.

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