

Application of an oxidative-biological treatment strategy for production of lactic acid and biomass from vinasse of sugarcane bioethanol industry

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ABSTRACT

In this study, the cultivation of lactic acid bacteria was done using vinasse (with a chemical oxygen demand of 378 ± 5 g O₂/L) to harness its organic content. The potential for biomass and lactic acid production was evaluated by using strains *Lactococcus lactis* subsp. *Cremoris* and *Lactococcus lactis* subsp. *Lactis*. Before medium preparation, vinasse was pretreated with air and ozone to reduce inhibitory load. The effects of pretreated vinasse addition on lactic acid bacteria growth were in the range of 0–33% v/v. The optimal vinasse concentration for obtaining high biomass and lactic acid concentrations was 17% v/v, leading to maximum concentrations of biomass and lactic acid of 2.2 ± 0.14 and 16.0 ± 0.9 g/L, respectively. Fed-batch operation was also studied as a strategy for extending the production phase of lactic acid bacteria using vinasse (17% v/v) as feeding. These results highlight the underexplored potential of vinasse as an economical source of raw material for obtaining value-added products through biotechnological processes.

1. INTRODUCTION

Anthropogenic climate change and overexploitation of natural resources, stemming from the continuous growth of the global population and industrial development, encourage the adoption of more sustainable and environmentally friendly processes. Regarding this, biofuels have been a part of the agenda for mitigating emissions and decreasing the dependence on fossil fuels. The global bioethanol production has increased by 30% in the last decade, reaching a value of 111–120 billion liters in 2023 [1]. Such bioethanol production might generate up to 1 trillion liters of vinasse worldwide, considering that the production rate of vinasse is variable, depending on the raw material, distillation methods, and distillation bottom handling [1,2].

In Colombia, the sugar industry developed several sugarcane varieties that are among the most productive in the world, harvested mainly in the upper valley of Cauca River (the second largest in the country). In this region, 456 million liters of ethanol are produced annually, generating up to 1.4 trillion liters of vinasse, a waste stream that imposes environmental pressures in industrial areas, primarily due to its handling and disposal [3,4]. In Colombia, bioethanol lies on

final molasses after sucrose crystallization (comprising 60–70% of the raw material), along with a portion of B molasses (30–40%) [4]. Vinasse can be characterized by the high chemical oxygen demand (COD) ranging from 70 to 120 gO₂/L, an average pH of 4.0, significant amounts of suspended solids, and honey/malt-like taste and smell [5,6]. Due to its composition, vinasse is considered to be approximately 100 times more contaminating than domestic wastewater [5,6]. Colombia lacks specific legislation regarding vinasse treatment, reutilization, and disposal. Vinasse is commonly employed for fertigating sugarcane, avoiding direct discharges into water bodies, but such application in improper doses may lead to lixiviation of high-organic-content liquids, soil salinization, and ion leaching, as remarkable environmental risks [3,4].

In contrast to the leading bioethanol producer in Latin America (Brazil), in Colombia, lower volumes of vinasse are produced by following an evaporation process to facilitate handling and transportation. Locally, 1–3 l of concentrated vinasse is generated per liter of anhydrous ethanol. The composition of this concentrated vinasse ranges between 20 and 50 °Brix, with high levels of potassium (0.6–3.0 kg K₂O/m³), nitrogen (0.57–1.2 kg/m³), and phosphorus (0.1–0.34 kg/m³) [4]. The evaporation of dilution water in vinasse is difficult; hence, vinasse is reused in biotechnological applications such as composting or anaerobic digestion, making their subsequent utilization more challenging [7]. In addition, the color of vinasse is of huge concern

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because some dyes are not only recalcitrant to biodegradation but are also inhibitors of biological activities [8,9].

Since fertigation of sugarcane crops may lead to soil saturation and contamination of water bodies, several methods have been explored for the treatment and utilization of vinasse, such as photocatalytic oxidation with ultraviolet light [10,11], the Fenton process based on the combination of iron salts with hydrogen peroxide, and the oxidation of recalcitrant compounds like melanoidins with ozone, all intended to decrease the COD [12]. Vinasse, due to its high carbon content, also represents a viable alternative for obtaining biogas for energy recovery through anaerobic digestion to produce methane and hydrogen as gases for combustion [13,14]. Despite the promising research, a significant and economically feasible reduction in the pollutant components has not been achieved, and, therefore, its final disposal in crop irrigation continues to be implemented as the only method [15,16].

Vinasses can be considered a valuable nutrient source for microbial growth given the high content in carbohydrates [17]. Growth of microalgae *Micractinium* sp. *Embrapa* LBA32 and *C. biconvexa* *Embrapa* LBA40 was tested by varying the vinasse concentration and supplementing the medium with 46–76% of carbon source [18]. Filamentous fungi of the *basidiomycete ligninolytic* family have shown great potential for degrading recalcitrant compounds by diluting vinasse up to 25% v/v [19]. Lactic acid bacteria (LAB) are grown using a mixture of vinasse of low sugar concentration (1.57% w/w) and sugar beet molasses with a high content of carbohydrates (53.16% w/w). In this case, vinasse was used as a solvent in the medium preparation [20].

LAB represent a significant group of microorganisms with wide industrial applications and are utilized extensively in the food and beverage industry, hence producing valuable products like lactic acid (LA), bacteriocins, and exopolysaccharides [20,21]. In recent years, there has been a notable focus on fermentative LA production, driven by the growing demand due to its various applications in different industries such as chemical, food, pharmaceutical, cosmetic, and polymer [22]. LA has multiple uses, serving as an acidifying and flavoring agent in food products, an antimicrobial substance and preservative in the cosmetic and food industries, and a precursor of the biodegradable polylactides in the pharmaceutical and plastics industries [23].

The global production of LA is approximately 270,000 tons per year, with 90% being produced through fermentation with pure substrates [24,25]. Despite various feedstocks utilized for LA production, significant challenges persist in achieving an economically viable production. Raw material substrates and fermentation processes constitute approximately 40–70% of production costs, and currently, LA production relies on costly sugars, often competing with food resources such as refined sugars (glucose) or starches [21]. Therefore, employing biorefinery platforms for waste materials generates high-value bioproducts while simultaneously addressing waste remediation. To address this issue, the use of low-cost substrates is strongly advocated for LA production [21].

While the adaptation of microorganisms to high concentrations of specific sugars or fermentation inhibitors has been proposed to improve bioethanol and xylitol production, data regarding the use of LAB for LA production using substrates rich in sugars and inhibitors, like in the case of vinasse, are still lacking [20]. The present study explores the pretreatment and subsequent use of vinasse as a substrate for the growth of LAB as an alternative valorization method for this

residue with high pollution potential and low demand in the market. Specifically, a consortium of LAB formed by *Lactococcus lactis* subsp. *Cremoris* (ATCC 19257) and *Lactococcus lactis* subsp. *Lactis* (ATCC 7962) was considered for lactic fermentation of vinasse for LA production.

2. MATERIALS AND METHODS

2.1. Characterization and Pretreatment of Industrial Vinasse

The vinasse used in this study was obtained from a sugar mill and distillery located in the city of Palmira, Valle del Cauca, Colombia. Vinasse in the distillery is the by-product of the bioethanol distillation after the fermentation of B-molasses. Distillation bottoms undergo concentration in a series of evaporators until reaching 32.7 ± 0.1 °Brix. The physicochemical characterization of vinasse was performed using the standard analysis methods, namely, AOAC 942.05 for ashes, AOAC 981.10 for nitrogen content, Lane–Eyn method for sugars determination, 5310 B high-temperature standard method for total organic content (TOC), ED 23/5220 C standard method for COD, and spectrophotometric Folin–Ciocalteu method for total polyphenol content.

For the pretreatment, the concentrated vinasses (2.0 l per batch) were decanted for a period of 24 h to remove settleable solids, which represented 15% of the sample volume. The decanted vinasse was diluted with distilled water at a ratio of 1:3 to approach the original concentration at which the effluent leaves the distillation tower in the industry. Finally, the pH was adjusted to 8.0 with 20% NaOH w/v. Subsequently, the liquid was aerated for 4 h at a flow rate of 0.27 l of air/(l of vinasse.s) for promoting the oxidation of ferrous ions (Fe^{2+}) and applying pH correction in the range of 8.0–9.0 by adding 20% NaOH w/v. To precipitate oxidized species, a second decantation of 18 h was applied. In addition, oxidative ozonation treatment at a low flow rate (28 mg O_3 /(l of vinasse.min)) for 4 h was applied to reduce the concentration of polyphenolic compounds.

2.2. Seed Medium and Microorganisms

In this study, a consortium of LAB consisting of *L. lactis* subsp. *Cremoris* ATCC 19257 and *L. lactis* subsp. *Lactis* ATCC 7962 was used. Both are subspecies of *L. lactis*, sharing many common metabolic properties, although they exhibit some differences in their metabolic profiles.

The microorganisms were cryopreserved at -16°C before being activated at 41°C in standard nutrient broth for LAB (MRS) for 18 h at 100 rpm on an orbital shaker (Heidolph, Unimax 1010) prior to the inoculation of the culture [26]. The basal medium MRS was prepared as follows (concentrations in g/L): lactose 20.0, peptone 10.0, yeast extract 12.0, K_2HPO_4 2.0, CH_3COONa 5.0, $\text{C}_6\text{H}_8\text{O}_7 \cdot 2\text{NH}_3$ 2.0, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2, and $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 0.05.

2.3. LA Fermentation

The fermentations with LAB were conducted in 125.0-mL flasks with a medium volume of 50 mL at the time of inoculation. The medium composition consisted of diluted vinasse according to the concentration required in the experimental design as solvent for media preparation and 5% of LAB inoculum was used. The pH was adjusted to 6.50 with 2.0 N sulfuric acid. Then, the media were sterilized at 121°C for 30 min.

Cultures were carried out in batch or fed-batch mode. In the latter case, 250.0-mL flasks were used with a final medium volume of 160 mL, starting with 40 mL of medium as previously described. All operations were carried out at 41°C and 100 rpm. During fermentation, biomass samples were taken and pH was measured, being corrected in the range of 6.0–6.5 with 20% w/v NaOH.

2.4. Analytical Methods

The oxidizable compounds in pretreated vinasse were determined using the Folin–Ciocalteu method as total polyphenols, with gallic acid as a standard [27], measured at 728 nm in a UV-Vis spectrophotometer (JASCO, V-730). The total reducing sugar content was determined using the Lane–Eynon method, titrating the Fehling's solution with the hydrolyzed sample until obtaining a brick-red color as the analyte concentration is obtained by volumetric relationship [28]. Total iron was quantified by atomic absorption spectrometry (Thermo Scientific, iCE3000) measured at 248 nm by the SM 3111B standard method [29]. The concentration of ferrous iron (Fe^{2+}) was determined following the Colombian technical standard NTC 4754:2000 using phenanthroline and measuring at 510 nm [30]. Biomass was measured as cell dry weight after drying at 75°C for 24 h.

The total solid content was determined by gravimetry. The concentration of total nitrogen was quantified using the AOAC 981.10 method, in which the sample is digested, distilled, and titrated with boric acid using a Sher indicator [31]. The percentage of ash was determined using the gravimetric AOAC 942 05 method, in which the sample is introduced into a muffle furnace at 600°C for 2 h, comparing the initial and final weights. To determine the TOC, the standard method 5310B was employed [32]. The sample is injected into a heated reaction chamber that transforms it into CO_2 and H_2O . The CO_2 is carried and measured by an infrared analyzer (IR). The COD in the concentrated and diluted vinasse was determined by spectrophotometry at 610 nm according to the USEPA 410.4 method [33]. The processing involved digesting the sample with oxidizing reagents at 150°C for 2 h and subsequent reading on equipment with an incorporated calibration curve.

3. RESULTS AND DISCUSSION

3.1. Effect of Pretreatment on Vinasse Inhibitor Concentration

Table 1 presents the results of vinasse characterization obtained from the last evaporation cycle in which vinasse by-product of the bioethanol distillation is concentrated up to 32.7 ± 0.1 °Brix. For utilization in microbiological cultures, vinasse was autoclaved as described earlier. It is important to highlight that vinasse in the distillery has been subjected at temperatures close to those arisen in an autoclave. Since the vinasse has suffered an extensive thermal process, it is expected that most of the temperature-sensitive compounds have already reacted, and, therefore, the composition before and after autoclaving remained almost constant. In this case, the registered variation in the total sugar concentration and total dissolved solids were lower than 5.0%.

As observed in Table 2, vinasse from the sugar industry contains important amounts of inhibitory compounds for the microbial activity, such as metals (mainly ferrous iron, Fe^{2+}) and polyphenols [8,34]. To mitigate the impact of these species, a preliminary treatment stage was conducted to vinasse diluted 1:3, that is, simulating the composition at the outlet of the distillation tower. In the pretreatment, vinasse was aerated from the bottom with an upward airflow; oxygen, upon contact

Table 1: Crude and autoclaved vinasse composition.

Component	Crude	Autoclaved
Total ash (% m/m)	8.16	8.04
Total nitrogen content (% m/m)	0.50	0.49
Total carbohydrates (% m/m)	27.73	28.587
Ethanol (mg/L)	0.64 ± 0.32	0.56 ± 0.41
Glycerol	8.39 ± 0.50	8.72 ± 0.22
Iron (mg/L)	108.56 ± 3.66	106.40 ± 2.98
Total organic carbon (g C/L)	129.84	128.32
COD (gO_2/L)	341 ± 4	339 ± 4
Total solids (% m/m)	29.90	30.23
Total polyphenols (g/L)	18.84 ± 2.36	18.98 ± 2.70
Density (g/L)	1143 ± 1.0	1160 ± 1.0
Acidity (pH)	4.74 ± 0.05	4.71 ± 0.05
Dissolved solids (Brix)	32.7 ± 0.1	33.11 ± 0.1

Table 2: Summary of general kinetic parameters of LAB batch fermentation with vinasse (17% v/v) and MRS medium.

Parameter	Symbol (Units)	Value
Fermentation time	t (h)	12
Substrate concentration	S ($\text{g/L}=\text{L}$)	23.40
Maximum biomass concentration	X_{\max} (g/L)	2.20
Maximum specific growth rate	μ_{\max} (h^{-1})	0.28
Maximum product (lactic acid) concentration	Q_{\max} (g/L)	16.01
Lactic acid yield	$P_{Q/S}$ (g/g)	0.59
Ratio of products	Q/X (g/g biomass)	7.27
Overall yield	$P_{P/S}$ (g/g)	0.67

with ferrous ions (Fe^{2+}), oxidizes them to ferric species (Fe^{3+}), which, combined with oxygen, results in iron oxide (Fe_2O_3), an insoluble species under basic pH. In addition, hydroxyl ions (OH^-) trap free ferric ions in the solution, forming iron oxyhydroxide ($\text{FeO}(\text{OH})$), another compound insoluble under these conditions, facilitating its removal by decantation [35]. The wine lees consisted mostly of organic matter 4.96 ± 1.20 g/kg, which includes yeast and remaining sugars. It also contains residual amounts of inorganics (in g/kg): nitrogen 0.70 ± 0.20 , phosphorus 0.90 ± 0.30 , potassium 11.70 ± 1.40 , magnesium 1.10 ± 0.20 , and iron 0.19 ± 0.04 . Such composition gives certain nutritional value to the lees for exploration of further valorization alternatives.

The Fe^{2+} concentration dynamics is presented in Figure 1. The dynamics of the pH serves as a proxy of the extent of reaction, as observed in the time course of ferrous ion in connection with the pH change. The aeration of the medium decreases the pH of the solution as the oxidation of the ferrous species advances, generating hydronium ions (H_3O^+) in the aqueous solution that are easily measurable [35]. Thus, the dynamics of the treatment can be followed online by pH measurement instead of sampling for Fe^{2+} (or Fe^{3+}) determination. According to the reaction dynamics, the optimum treatment time is estimated in 2.2 h as the rate of change slows down after 2 h of aeration.

It has been reported that the oxidation of Fe^{2+} proceeds more efficiently in a basic medium above pH 8.0 since it favors the kinetics of oxidation

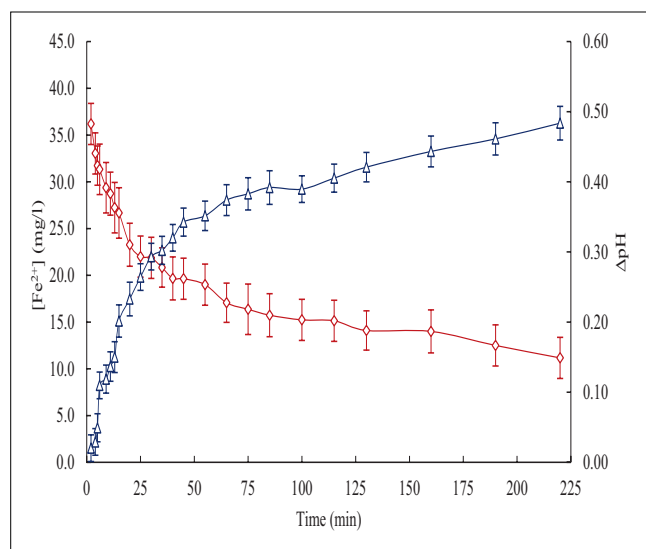


Figure 1: Ferrous ion concentration (diamonds) and Δ pH (triangles) time course during vinasse (33% v/v) pretreatment with airflow of 0.27 l of air/(l of vinasse.s).

and increases the stability of the formed molecule [36]. In this case, to enhance the oxidation of aqueous iron, the pH was adjusted every 8 min for controlling between 8.0 and 9.0 by adding 20% w/v NaOH. This facilitated the formation and precipitation of ferric ions as iron oxide (Fe_2O_3) [36]. The application of aeration as pretreatment led to a 26% decrease in oxidizable compounds in solution, consequently reducing the inhibitory load of vinasse before its use as a substrate for fermentation with LAB.

Aeration reduced the Fe^{2+} content in the vinasse down to 68%, one of the main inhibitors of the microbial activity in the vinasse. Since polyphenols are organic compounds difficult to oxidize by aeration, the addition of a subsequent ozonation stage was proposed to increase the proportion of inhibitors eliminated from the raw diluted vinasse. The ozonation stage contributed to a 22% reduction in polyphenols from 6.28 ± 1.35 to 4.90 ± 1.84 g/L, leading to a total decrease of 48% in the concentration of oxidizable species in vinasse.

LAB cultures in media prepared with pretreated vinasse and MRS components were compared at concentrations ranging from 1 to 10% v/v of final vinasse concentration to visualize the effect of treatment in the maximum biomass accumulation. As shown in Figure 2, the differences in biomass concentrations obtained in each medium ranged from 0.1 to 0.4 g/L, increasing the biomass as the fermentable sugar concentration increases as a result of higher proportion of vinasse in the final medium.

The high concentration of oxidable inhibitors in the crude diluted vinasse did not allow any appreciable growth of LAB at all dilutions (negative control). As expected, in all cases the maximum biomass was observed in the medium with lower inhibitor concentration (vinasse aerated and ozonized). The average biomass production using vinasse treated with air/ozone is 11% higher than the vinasse treated only with air. Therefore, both pretreatments improve the conditions for using vinasse as a substrate or supplement for LAB fermentation. Nevertheless, the cost of applying ozonation is considerably higher compared to the simple aeration [37]. This vinasse has a low content of ethanol, as shown in Table 1. The content of ethanol is 0.56 ± 0.41

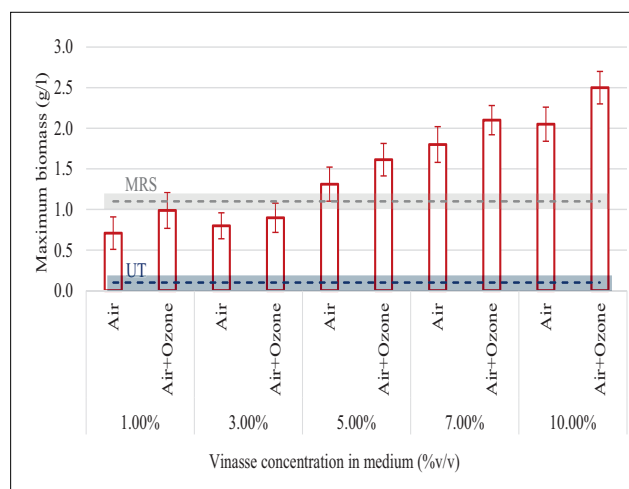


Figure 2: Biomass (bars) of lactic acid bacteria cultivated in pretreated vinasse (1–10% v/v) with MRS medium. Positive control: pure MRS medium (gray line). Negative control: untreated vinasse (blue line).

mg/L, and the variability in ethanol composition did not correlate with the biomass variability in control experiments (Pearson correlation test, p-value = 0.2191). Since biomass values did not show significant differences between pretreatments at all dilutions, it was decided to consider only aeration as the most economically feasible pretreatment. This is advantageous for application in the industrial scale, where the less sophisticated and cheap waste treatments are preferred. Thus, the simple aeration is a promising technique for decreasing the inhibitor composition in the distillation effluent for further utilization of vinasse in biological processes.

3.2. Effect of Vinasse on Lactic Acid and LAB Growth

Ethanol producers in the region of Valle del Cauca perform concentration of distillation bottoms to reduce the overall volume of vinasse in the plant, resulting in an approximate ratio of 1–3 l of vinasse per liter of anhydrous bioethanol for fuel mixtures [3]. Without concentration, this ratio reaches up to 10 l of vinasse per liter of distilled ethanol [38]. Under these considerations, LAB biomass and LA production were explored using pretreated vinasse for medium preparation with final concentrations ranging from 0 to 33% v/v. Figure 3 displays the LA and biomass maximum concentrations attained at 24 h of batch cultivation between 0 and 20% v/v vinasse concentration in the medium. Vinasse concentrations over 20% totally inhibited the LAB growth (data not shown).

The data summarized in Figure 3 evidence a maximum point of biomass and LA accumulations when cultivated using a medium prepared with vinasse up to 17% v/v, doubling the value obtained when using standard MRS medium. However, as the vinasse concentration increases beyond 17% v/v, biomass and LA production decreases, suggesting that inhibitory compounds became significant at less than 20% v/v and higher. Since the purpose is to treat the vinasse at the highest viable concentration promoting high LAB growth and LA accumulation, the 17% v/v dilution was established as the most suitable concentration for further application. Moreover, by dealing with this concentration, it does not necessarily imply the use of fresh water for dilution since the concentration of vinasse varies according to the fermented molasses fed to the distillation tower. Vinasses can be obtained from the first, second, or third centrifugation stage as A-, B-,

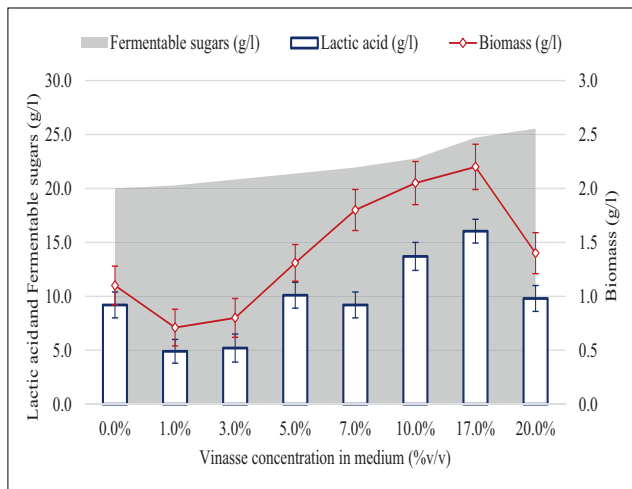


Figure 3: Lactic acid, fermentable sugars, and biomass maximum concentrations in 24 h batch cultivation using media prepared with pretreated vinasse.

and C-type molasses, respectively, conferring variable characteristics of vinasse leaving the distiller [39]. In addition, evaporation processes would be unnecessary in a biological treatment scenario, reducing operating costs related to the required thermal energy.

To analyze bacterial growth under the established culture conditions as experimental optimum, biomass and LA were monitored during the complete fermentation. Samples were taken and pH was adjusted by adding 20% w/v NaOH. The results show that maximum biomass is reached at 12 h with a maximum specific growth rate (μ_{\max}) of 0.28 h^{-1} , which aligns with the reported range in previous studies estimating a time between 10 and 12 h [21,40]. The maximum LA accumulation was recorded between 14 and 20 h.

The growth curve of LAB [Figure 4] confirms the kinetics of biomass, substrate uptake, and metabolite production. The secretion in the fermentation medium causes a decrease in pH, which is related to the concentration of acid and its conjugate base through the proton mass balance (H_3O^+) [41,42]. This also can act as an inhibitor of the bacterial growth, so the pH control can extend the stationary phase of the culture. Therefore, the progress of the culture can be easily followed online through two main variables (biomass and pH), providing certainty about the direction of the fermentation process and metabolite production.

LAB can ferment not only the lactose used as carbon source in the MRS medium but also may use the sugars present in the vinasse to produce LA and biomass as main products given their homofermentative nature. In addition to lactose, these strains can use other sugars such as glucose, galactose, and sucrose as carbon sources, as well as peptides and amino acids as nitrogen and energy source, expanding their potential in various biotechnological applications [43,44]. The results in Figures 3 and 4 confirm that increases in the fermentable sugars in the media lead to more LA and biomass being produced, approaching the theoretical yield of 1.0 g LA/g of fermentable sugars in the medium [45].

Table 2 summarizes the kinetic constants of batch cultivation of LAB key aspects of the fermentation process. Initially, it was observed that the total duration of fermentation is 12 h. During this process,

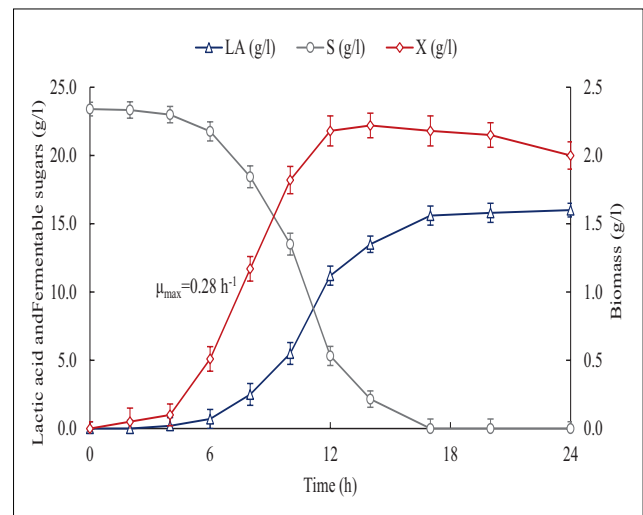


Figure 4: Lactic acid (LA), fermentable sugars (S), and biomass (X) time course of concentrations in LAB cultures with 17% pretreated vinasse.

it starts with an initial concentration of available sugars of 31 g/L, resulting in an overall yield of 0.67. This value is very similar to previous reports on pure cultures with LAB [46]. For comparison, a yield of approximately 78% in a standard medium *L. lactis* is reported. The difference between these two values is only 14%, supporting the good performance obtained, considering that vinasses are residual and complex substrate for the culture of any microorganism, given the significant imbalance in salts content and oxidable compounds of inhibitory nature.

Given the interest in mitigating the environmental impact associated with vinasse management, evaluations of variables related to discharge and post-treatment disposal were conducted [47]. In the first stage of pretreatment, a significant 14% reduction in COD was achieved, starting from a relatively high value of $341 \pm 4 \text{ gO}_2/\text{L}$ of solution. This decrease had a significant effect on the growth of microorganisms, as described previously, suggesting that different microorganisms present in the environment may find favorable conditions to consume nutrients and degrade the pollutants in the distillation effluent pretreated with simple aeration. After the combined oxidative and biological treatment, this value decreased to $156 \pm 4 \text{ gO}_2/\text{L}$. In addition, the pH was adjusted to 6.50, complying with environmental regulations established in Resolution 0883 of 2018 issued by the Ministry of Environment and Sustainable Development of Colombia [48], which set a range between 6.0 and 9.0 pH units for effluent discharge. This progress is significant, considering that the initial pH of the vinasse is 4.74 ± 0.05 . Another crucial aspect of the process was the complete removal of total suspended solids in the final stage. These results indicate significant progress in treating vinasse as a contaminant, favoring the possibility of using it more safely and sustainably. Further improvements are needed as the implementation of a purification strategy to obtain D-lactic acid with high purity degree, and the possible utilization of this effluent in anaerobic digestion and composting, in order to get valuable products like hydrogen and biogas. After such a combination of treatments, the pollution potential of the vinasse will be likely reduced almost completely.

3.3. Setup of Fed-batch LAB Cultivation Using vinasse

Considering the high volume of vinasse generated in the region, fed-batch operation was proposed as an alternative to increase the utilization of this residue as substrate for LAB cultivation. For this purpose, vinasse was added after 12 h of fermentation in pulses to observe the response of LAB to vinasse addition. The results presented in Figure 5 evidence a sustained growth over 48 h, in contrast to that observed in batch operation [Figure 4]. The dilution effect due to medium addition after the 24th h decreases the biomass concentration slightly [Figure 5A]. Nevertheless, the biomass concentration remains almost constant during the fed-batch phase as the dilution is approximately equal to the growth rate.

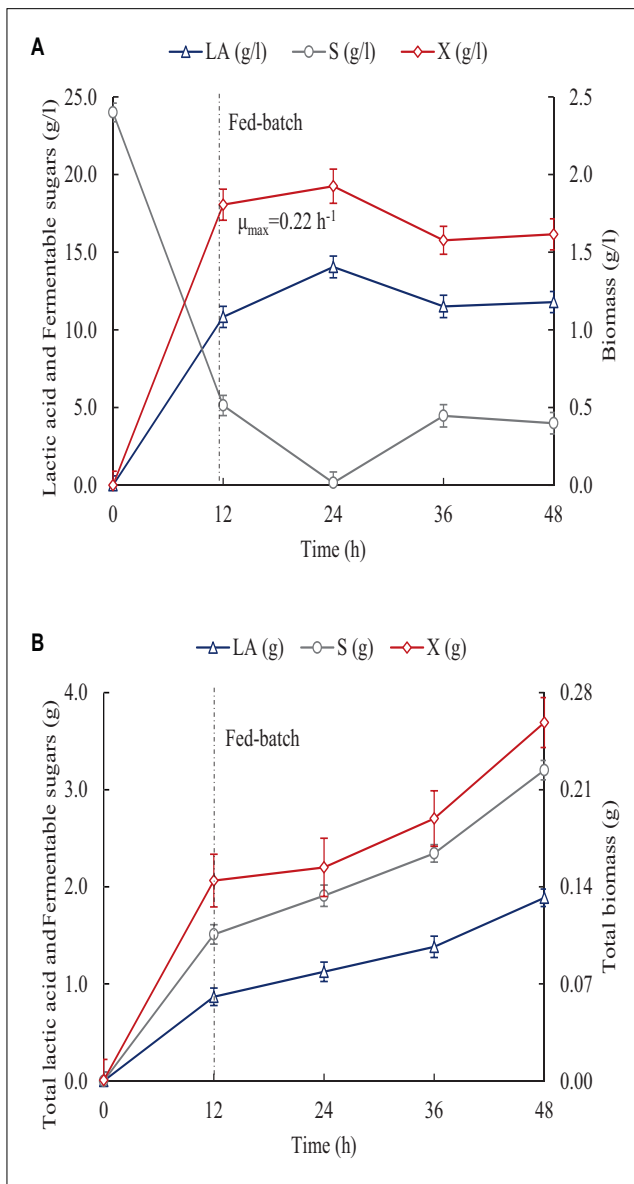


Figure 5: Lactic acid (triangles), fermentable sugars (circles), and biomass (diamonds) in LAB cultures with 17% pretreated vinasse. **(A)** Time course of concentrations. **(B)** Time course of substrate, biomass, and product accumulation.

In Figure 5B, the total accumulation of LA, biomass, and fermentable sugars is presented. During the first 12 h, a significant increase in biomass characteristic of the exponential growth ($\mu_{\max} = 0.22 \text{ h}^{-1}$) phase is noted, where the nutritional factors supplied by the components of the MRS medium promote the acceleration of biomass production. The addition of vinasse as a supplement in the fed-batch stage provides fermentable sugars but also increases the composition of inhibitors, but those apparently do not affect the growth trend of LAB, in which the specific growth rate is 0.02 h^{-1} during the deacceleration phase. The contribution of carbohydrates in the vinasse serves as a carbon source to sustain a growth phase with a constant supply of those nutrients available in the $40.0 \pm 0.5 \text{ mL}$ of 17% v/v vinasse in each addition.

These results suggest that it is feasible to carry out fed-batch operation in the cultivation of LAB using a medium that includes vinasse as a supplement. The findings of this research offer promising prospects linked to the production of LA and biomass of LAB. In addition, as seen in other studies, the valorization of waste streams yields good performances in relation to LA production [46,49]. Currently, the commercial production of LA is mainly carried out through bacterial fermentation of glucose derived from sources such as sugarcane or beet molasses, corn starch, rice, wheat, potatoes, barley, and cassava, posing competition for the food production as they are also used for human and animal consumption [24].

The possibilities of using pretreated vinasse with a simple and economical method like aeration add to other materials with potential, such as lignocellulosic materials (sawdust, poplar, sugarcane bagasse), and spent brewery grains, among others, which are considered promising raw materials for LA production. This is due to their abundance, potential cost-effectiveness, high carbohydrate content, and noncompetition with the food chain [19]. In addition, the biomass obtained in the process, when supplemented with organic sources like breadcrumbs or lentil residues, has the potential to be used as a probiotic in animal farming.

4. CONCLUSION

During the vinasse pretreatment stage, a notable 67% reduction in the total Fe^{2+} content in the medium was achieved through the aeration process. Subsequently, in the aeration-ozone stage, a 22% oxidation of polyphenols was attained. These results reduce the inhibitors of the bacterial growth sufficiently, allowing the proliferation of LAB compared with nontreated vinasse, thus supporting the implementation of these pretreatment procedures. However, it should be noted that the difference in biomass generated between aeration treatment alone and the combined aeration-ozone treatment is only 11%. This difference does not justify the adoption of the ozone process due to the associated implementation costs; therefore, cheaper aeration treatment is recommended.

The pretreated vinasse in combination with standard MRS medium proved to be a valuable nutrient source for the growth of LAB strains *L. lactis* subsp. *Cremoris* ATCC 19257 and *L. lactis* subsp. *Lactis* ATCC 7962. Maximum values of 16 g/L of LA and 2.2 g/L of biomass were achieved, approaching the theoretical yield of 1 g LA/g sugars despite the waste nature of the substrate. These results suggest promising prospects for the generation of value-added products from vinasse as a substrate. Further studies are required to generate tolerant strains that allow the utilization of more concentrated vinasses. Under the study's conditions, the treatment at vinasse concentration of 17% v/v is still

a high dilution rate (one part of distillation bottoms per one part of water), considering that the concentration of the distillation tower effluent is equivalent to a crude concentrated vinasse dilution of ~33% v/v. The adoption of biorefinery approaches integrating this treatment as part of a valorization chain considering biomass utilization in animal nutrition, recovery of LA as raw material, utilization of vinasse for production of other added-value compounds, and finishing with composting and biogas generation may offer a sustainable alternative for this effluent with significant environmental implications.

5. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

All data generated and analyzed are included in this research article. The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

10. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

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