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# Lactic acid production by *Carnobacterium* sp. isolated from a maritime Antarctic lake using eucalyptus enzymatic hydrolysate

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ABSTRACT

*Carnobacterium* sp., a lactic acid bacterium isolated from a maritime Antarctic lake, was evaluated for lactic acid production from a lignocellulosic hydrolysate. Eucalyptus sawdust, a residue from pulp and paper industries, was subjected to alkaline pretreatment to enhance its enzymatic hydrolysis. Fermentations were performed without and with pH control using eucalyptus enzymatic hydrolysate containing a mixture of glucose and xylose sugars. The sugars were successfully converted into lactic acid in 24 h, resulting in 7.6 g/L of lactic acid and a product yield of 0.50 g/g for pH controlled at 6.5. Fed-batch fermentation performed at a controlled pH of 6.5 improved both the lactic acid production (30 g/L) and the biomass growth (4.2 g/L). L-lactic acid optical purity higher than 95 % was obtained. These results demonstrated the potential usage of *Carnobacterium* sp in L-lactic acid production from eucalyptus.

## 1. Introduction

Lactic acid is one of the most extensively used chemicals today because of its applications in the food industry as a preservative, acidifier, and flavoring; in the textile and pharmaceutical industry; as well as in the chemical industry as a raw material for a wide variety of commercial value-added compounds [1]. In recent years, the production of lactic acid has gained increasing attention given its use in the synthesis of the biopolymer poly lactic acid (PLA).

Lactic acid can be produced through chemical synthesis or microbial fermentation. Some of the main disadvantages of chemical synthesis are the high cost of production and the use of various highly toxic compounds such as methanol and hydrocyanic acid [2]. Furthermore, a racemic mixture of D- and L-lactic acid is produced, which requires the usage of complex separation and purification techniques in the production of PLA, because high purity of the same isomer, D- or L-, is needed [3]. Compared to chemical synthesis, microbial production of lactic acid is a better alternative because only pure D-lactic acid or L-lactic acid can be produced depending on the enzyme lactate dehydrogenase used [4]. Although both pure isomers can be used to produce crystalline structures of PLA, the L-isomer is also useful for food applications since it is suitable for human consumption, in contrast to the D-isomer which can cause metabolic problems in human beings [1,5,6]. Moreover, microbial fermentation presents advantages in the utilization of renewable carbohydrates for lactic acid production. Due to these advantages over chemical synthesis, microbial fermentation accounts for 90 % of the global production of lactic acid [2].

Lactic acid bacteria (LAB) have been extensively used for lactic acid production. Among the bacterial genera belonging to LAB are *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* [7]. LAB are Gram-positive, aero-tolerant anaerobes, non-sporulating rods and/or cocci that have been isolated from different sources such as grains, green plants, fresh and decomposing sponges, seaweeds, shellfish, and fish in marine environments, among others [8]. They can be classified according to their fermentation patterns in the following three groups: homofermenters, which can metabolize hexoses through the Embden-Meyerhof-Parnas (EMP) pathway and having lactic acid as the main product; heterofermenters, which are able to metabolize hexoses and pentoses through the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway and produce lactic acid as well as carbon dioxide, ethanol and/or acetic acid; and facultative heterofermenters, which can use both pathways and usually ferment the hexoses through the EMP pathway as well as activate the 6-PG/PK pathway in the presence of pentoses [1,2,8–11]. *Carnobacterium* spp. have been isolated from less explored niches such as marine environments and coastal and estuarine sediments; they have also been associated with spoilage in meat at low temperatures. This LAB genus, classified as facultative

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heterofermenter, has been extensively studied in the last two decades due to the important role they play in the biopreservation of food products by inhibiting pathogenic and spoilage microorganisms, including potential spoilage bacteria in seafood and meat products. Additionally, due to their presence in aqueous environments, their importance as probiotic cultures in the aquaculture industry has been investigated [12–14]. In addition to the applications mentioned above, *Carnobacterium* spp. present some characteristics that make them interesting for its study for lactic acid production. These include the production of pure L-lactic acid [15], less fastidious nutrition requirements and higher tolerance to oxygen than homofermentative lactobacilli [16]. However, few studies on lactic acid production by fermentation of *Carnobacterium* spp. have been reported, probably due to the difficulty of isolating them compared to other LAB [15].

Different substrates have been used for the biotechnological production of lactic acid, including glucose, sucrose, lactose, maltose, mannose, xylose, and galactose, enabling the production of pure product and resulting in lower purification costs [7,9,11,17,18]. However, for the economic feasibility of biotechnological production, inexpensive lignocellulosic feedstock rather than costly refined substrates should be used [1]. The production of lactic acid has been studied using different lignocellulosic biomass types, such as sugarcane bagasse, cassava bagasse, wheat straw, corncobs, rice straw, soybean straw, barley bran husks, and elephant grass, among others [19–21]. Lactic acid fermentation from these materials consists of the sugar being released from the biomass cell wall by hydrolysis through pretreatment and saccharification, followed by fermentation. Studies demonstrate that an efficient bioconversion of lignocellulosic biomass into pure D- or L-lactic acid through LAB fermentation can be achieved through the selection of an optimal combination of pretreatment, saccharification, and fermentation process configuration, as well as a LAB strain suitable for a specific substrate [1,10]. For instance, for lignocellulosic substrates, a LAB strain fermenting both pentoses and hexoses is required to maximize the process yield.

In Uruguay, the eucalyptus wood represents a potential woody feedstock because of its availability and adaptability to the climate compared to other wood species. Most eucalyptus plantations in Uruguay are currently used for cellulose pulp production, which has rapidly increased and expanded in the last decades in South America. However, during the eucalyptus pulp production process, particularly during wood chipping, considerable amounts of solid residues (eucalyptus sawdust) are discarded from the pulping process due to size limitations. Moreover, it has been previously demonstrated by our research group that this material represents an attractive renewable resource in biorefineries for the production of biofuels and value-added platform chemicals [22–24]. The utilization of eucalyptus sawdust from local pulp industries as a substrate for lactic acid production has not been reported in the literature and is considered an interesting alternative to improve the pulp and paper industry revenues.

Beyond the LAB and nature of the substrate used, there are several factors that substantially affect the efficiency of microbial lactic acid production, such as pH and temperature of the culture medium, nitrogen and vitamin sources, operation mode of the fermentation, and byproduct formation [2]. pH is one of the main factors affecting lactic acid fermentation considering that the enzyme catalytic activity and metabolic activity of the microorganisms greatly depend on the extracellular pH [17]. pH control of the fermentation broth during the fermentation process through base titration or lactic acid removal (e.g., extraction, adsorption or electrodialysis) results in higher lactic acid production, yield and productivity [11,25]. Regarding the fermentation operational mode, although batch fermentations have been widely used due to higher production of lactic acid, it has been reported that continuous and fed-batch mode could achieve better production yields compared to batch mode [26].

Although high titers of lactic acid production are found in the literature using different microorganisms, pure substrates such as sucrose from sugarcane and sugar beet are needed to obtain a product with high purity which increases the production cost [2]. For this reason, the use of low-cost substrates, such as lignocellulosic biomass, has become a promising source for lactic acid production.

To the best of the authors' knowledge, there are no reported data on lactic acid production from lignocellulosic materials using *Carnobacterium* strains. The aim of the present work was to study the potential use of a psychrotolerant strain of the *Carnobacterium* genus isolated from a water sample from Uruguay Lake, King George Island, Antarctica for lactic acid production from a residue of the pulp and paper industries. The enzymatic hydrolysis of eucalyptus sawdust subjected to alkaline pretreatment at high solid concentrations using different enzyme loadings to maximize the release of the fermentable sugars glucose and xylose was evaluated. Moreover, this work investigated the effect of fermentation pH control and operating mode on the production of lactic acid by *Carnobacterium* sp, performing batch and fed-batch fermentations of the eucalyptus enzymatic hydrolysate without and with pH control. This work provides a first insight into the use of psychrotolerant *Carnobacterium* spp for L-lactic acid production from cheap renewable sources.

## 2. Materials and methods

### 2.1. Microorganism

*Carnobacterium* sp. was isolated from Uruguay Lake, King George Island, Antarctica. Uruguay Lake water samples were collected from a depth of 5−10 m. Approximately, 100 μL of the liquid samples were transferred into bottles with 50 mL of M141 liquid media prepared as described by Franzmann et al. [27] and incubated at 20 °C with orbital shaking at 150 rpm. Then, 100 μL of the grown M141 culture media were spread on De Man, Rogosa, and Sharpe (MRS) agar plates containing 20 g/L glucose, 10 g/L peptone, 10 g/L beef extract, 5 g/L yeast extract, 5 g/L sodium acetate, 1 g/L Tween 80, 2 g/L dipotassium hydrogen phosphate, 2 g/L ammonium citrate, 0.1 g/L magnesium sulfate, 0.05 g/L manganese sulfate, and 15 g/L agar. Bromocresol purple was used as the pH indicator (0.02 % w/v). The plates were incubated in anaerobiosis for 7–10 days at 20 °C. The colonies with a yellow halo around them were selected and isolated through repeated transfer on MRS media. The isolate was then identified via amplification of the 16S rRNA gene and sequencing (data not shown in this study). Stock cultures were prepared from the isolate, maintained in 20 % glycerol and stored at −80 °C.

### 2.2. Raw material

Eucalyptus sawdust, which was used as raw material, was provided by a local pulp mill (UPM Fray Bentos, Uruguay). The material was dried at 40 °C until 8% (w/w) moisture content was achieved, after which it was stored at room temperature. The particle size distribution of the material, determined through dry sieve analysis, was 4.9 % between 3.35 mm and 6.5 mm, 45.2 % between 1.40 mm and 3.35 mm, 37.3 % between 1.19 mm and 1.40 mm, 9.3 % between 0.5 mm and 1.19 mm, and 3.4 % below 0.5 mm. The material below 0.5 mm (fines) was discarded. Its composition is presented in Table 1.

### 2.3. Alkaline pretreatment of eucalyptus sawdust

The eucalyptus sawdust was subjected to alkaline pretreatment in a silicone oil bath heating circulator with eight stainless steel batch cylinder reactors (Fibretec Inc., India). The raw material was mixed with 15 % NaOH (on a dry biomass basis) at a liquid to solid ratio of 8 $g_{liquid}/g_{dry\ eucalyptus}$ in the reactors with a nominal volume of 300 mL. The silicone oil bath was first pre-heated to 100 °C before the reactors were submerged. The system was heated to a maximum temperature of 160 °C in 45 ± 5 min and then kept at maximum temperature for 45 min (H-factor

**Table 1**
Chemical composition of untreated and alkaline-pretreated eucalyptus sawdust.

| Component | Weight percent (%, on dry wood basis) |
|---|---|
| *Raw material* | |
| Glucan | 45.4 ± 0.4 |
| Xylan | 13.4 ± 0.3 |
| Acetyl | 2.3 ± 0.1 |
| Acid insoluble lignin | 24.2 ± 0.7 |
| Acid soluble lignin | 4.5 ± 0.6 |
| Extractives | 7.1 ± 1.1 |
| Ash | 0.7 ± 0.3 |
| *Alkaline-pretreated solid* | |
| Glucan | 58.0 ± 3.1 |
| Xylan | 13.1 ± 0.5 |
| Acetyl | nd |
| Acid insoluble lignin | 20.2 ± 0.1 |
| Acid soluble lignin | 3.8 ± 0.1 |
| Ash | 0.3 ± 0.1 |
| Solid yield | 68.2 ± 1.2 |

*nd*: not detected.

of 522). After pretreatment, the reactors were removed from the silicone bath and cooled with tap water for 10 min before separating the solid and liquid fractions. The solid fraction was extensively washed with distilled water by centrifugation until a pH close to neutrality was achieved. The washed solid fraction (alkaline-pretreated eucalyptus) was characterized for total solids, carbohydrates and lignin, after which it was stored at 4 °C for the enzymatic hydrolysis assays.

### 2.4. Enzymatic hydrolysis of alkaline-pretreated eucalyptus sawdust

Enzymatic hydrolysis assays of the alkaline-pretreated eucalyptus sawdust were carried out at 50 °C and pH 4.85 (0.05 N citric acid-sodium citrate buffer) in 250 mL Erlenmeyer flasks with orbital agitation at 150 rpm. The enzyme used was the commercial cellulase preparation Cellic CTec2 with a cellulase activity of 163 FPU/mL, determined according to NREL protocol [28]. The effects of solid concentration and enzyme loading were evaluated using an experimental three-level factorial design with two factors ($3^2$, total of nine experiments). Hydrolysis assays were performed in duplicate at solid concentrations in the range of 4–10 % (w/w) and enzyme loadings in the range of 10–20 FPU/$g_{glucan}$. Samples were collected periodically, heated at 100 °C for 10 min to deactivate the enzymes, and then centrifuged at 15,600 *g* for 10 min. Supernatants were collected after centrifugation for glucose and xylose determination. Glucose concentration in the supernatants and enzymatic hydrolysis yield were used to select the best enzymatic hydrolysis conditions regarding hydrolysis time, solid concentration, and enzyme loading.

### 2.5. Inoculum preparation for fermentations

Fermentation inocula were prepared in 250 mL bottles with 200 mL of MRS medium. The pH of the medium was adjusted to 6.5 ± 0.1. The medium was swept with $O_2$-free $N_2$ over the headspace of the bottles, and then sterilized at 121 °C for 15 min. Upon cooling to room temperature, inoculation took place with the cells previously grown in MRS medium from stock cultures and incubated at 20 °C and 150 rpm for 24 h. The biomass for inoculation was grown at 20 °C and 150 rpm for 72 h.

### 2.6. Batch and fed-batch fermentation

The lactic acid fermentations were performed in a 5 L bioreactor Biostat A Plus (Sartorius) with 1.5 L of fermentation medium at 20 °C and 150 rpm. The fermentation media consisted of modified MRS medium, substituting the carbon source of the MRS by eucalyptus enzymatic hydrolysate as the carbon source. The pH of the media was adjusted to 6.5 ± 0.1. The fermentation media were swept with $O_2$-free $N_2$ over the headspace of the bioreactor, and then sterilized at 121 °C for 15 min. Upon cooling to room temperature, inoculation took place with the highly active cells that were grown in the inoculum MRS medium to obtain an initial biomass concentration of about 0.1 g/L in the fermentation broth.

Batch fermentations were carried out without and with pH control. In the second case, the pH was set at 6.5 and controlled with the addition of $H_2SO_4$ (2.5 M) or NaOH (2.5 M). In the fed-batch fermentation, a concentrated pulse of glucose (225 g/L), xylose (50 g/L), beef extract (75 g/L), peptone (75 g/L), and yeast extract (37.5 g/L), was added to the fermentation broth when glucose has been completely consumed (32 h of fermentation) to reach a total sugar concentration of 60 g/L. The pH was controlled at 6.5. Samples were taken periodically to monitor cellular growth, substrate consumption, and lactic acid production.

### 2.7. Calculations

The yield coefficient for lactic acid production on total sugar consumption, $Y_{P/S}$ ($g_{lactic\ acid}/g_{sugars}$), and the yield coefficient for biomass production on total sugar consumption, $Y_{X/S}$ ($g_{biomass}/g_{sugars}$), are expressed by Eq. 1 and Eq. 2, respectively:

$$Y_{P/S} = m_P/(m_{S,0} - m_S) \quad (1)$$

$$Y_{X/S} = \frac{(m_X - m_{X,0})}{(m_{S,0} - m_S)} \quad (2)$$

where $m_P$ and $m_X$ are the amount of lactic acid and biomass produced, respectively, $m_{S,0}$ and $m_{x,0}$ are the initial sugar and biomass amount in the culture broth, respectively, and $m_S$ is the sugar amount remaining at the end of the fermentation ($m_p$, $m_X$, $m_{S,0}$, $m_{X,0}$ and $m_s$, expressed in g).

The total volumetric lactic acid productivity ($Q_p$) was given by the final lactic acid concentration divided by the fermentation time (t, expressed in h).

$$Q_P = P/t \quad (3)$$

The efficiency of lactic acid production is expressed by Eq. 4:

$$\eta = Y_{P/S} \,/\, Y_P \quad (4)$$

where $Y_P$ is the theoretical coefficient for lactic acid production calculated based on the initial proportion of glucose and xylose in the fermentation broth and the theoretical coefficients for lactic acid production from glucose (1.0 $g_{lactic\ acid}/g_{glucose}$) and xylose (0.6 $g_{lactic\ acid}/g_{xylose}$).

Sugar conversion ($x_S$) is expressed as the percentage of total sugar that was effectively consumed by the microorganism, as shown in Eq. 5:

$$x_S = (S_0 - S)/S_0 \times 100 \quad (5)$$

where $S_0$ is the initial sugar concentration (g/L) and S is the final sugar concentration (g/L) in the fermentation broth.

### 2.8. Analytical methods

#### 2.8.1. Chemical composition of eucalyptus sawdust

The chemical composition of the untreated and alkaline-pretreated raw material was determined according to NREL protocols as follows: carbohydrates and lignin [29], ash [30], and extractives [31].

#### 2.8.2. Determination of sugars, byproducts, and products

The sugars (xylose, glucose), byproducts (acetic acid and formic acid) and products (lactic acid) concentrations in supernatants from the enzymatic hydrolysis and fermentation assays, when corresponded, were determined through HPLC. An HPLC Shimadzu instrument (Kyoto, Japan) equipped with a refractive index detector (RID-10A) and a

Biorad Aminex HPX-87H column were used for the determinations. The HPLC conditions were 0.005 M sulfuric acid as mobile phase, flow rate of 0.3 mL/min, column temperature of 45 °C, and injected volume of 20 μL. The optical purity of lactic acid was assayed using the D- and L-lactic acid kit (Megazyme, Bray, Wicklow, Ireland).

#### 2.8.3. Biomass concentration

The biomass concentration was determined by optical density at 600 nm using a spectrophotometer Genesys 10S UV–vis (Thermo Scientific). The samples were diluted with distilled water to obtain absorbance values lower than 0.8. The relation between optical density (OD) and dry cell weight (DCW) was determined, and biomass estimation was reported as g dry cell per liter of culture medium (g/L), considering that one unit of OD corresponded to 0.71 ± 0.03 g/L of DCW.

### 2.9. Statistical analysis

Analysis of variance (ANOVA) studies were performed to ascertain if the differences found were statistically significant ($p \leq 0.05$). The software used in this analysis was InfoStat (student version 2019). The Tukey's test was used to find the significant differences between means.

## 3. Results and discussion

### 3.1. Eucalyptus sawdust composition and alkaline pretreatment

Eucalyptus sawdust proved to be an abundant source of carbohydrates (58.8 %), mainly glucan (45.4 %), according to its chemical composition (Table 1). However, a considerable amount of lignin (28.7 %) was also found in the biomass, which corresponds quite well with previously reported data [22,24]. Since this high lignin content may compromise enzymatic hydrolysis efficiency, alkaline pretreatment was carried out in this work to enhance the delignification of biomass. In this study, eucalyptus sawdust was pretreated with 15 % NaOH because it was the condition assessed that maximized the enzymatic hydrolysis of pretreated eucalyptus sawdust, according to previous work (data not shown). According to the results obtained, alkaline pretreatment effectively removed a significant amount of lignin (43 %) and hemicellulose (33 % and 100 % for xylan and acetyl groups, respectively) from eucalyptus sawdust, which allowed glucan concentration to reach 58 % in the pretreated sawdust. Similar results were also achieved by Carvalho et al. [32] under similar alkaline pretreatment conditions (15 % NaOH, 175 °C, H-Factor 628) using eucalyptus wood in terms of lignin and hemicellulose removal (51 % and 61 %, respectively), and glucan recovery (90 %).

### 3.2. Effect of solid concentration and enzyme loading on fermentable sugars production

The alkaline-pretreated eucalyptus sawdust was then subjected to enzymatic hydrolysis at 4–10 % solid concentrations using different enzyme loadings (10–20 FPU/$g_{glucan}$) to maximize the fermentable sugars released for lactic acid production. After the experimental design, the average results of the experiments in terms of fermentable sugar concentrations and enzymatic hydrolysis efficiency are shown in Figs. 1 and 2, respectively. Notably, increasing the solid concentration improved glucose and xylose concentrations in the eucalyptus enzymatic hydrolysate much more than increasing the enzyme loading. There were sugar concentration increments of 40%–56% when the solid concentration increased from 4% to 10 %. However, the increment percentages (24%–50%) were lower when enzyme loading increased from 10 FPU/$g_{glucan}$ to 20 FPU/$g_{glucan}$. It should be noted that a considerable amount of xylose (3.8–9.4 g/L) was also produced during the enzymatic hydrolysis of alkaline-pretreated sawdust owing to the relatively low xylan solubilization caused by the pretreatment. On the other hand, as expected, hydrolysis efficiency increased with enzyme loading but decreased with solid concentration, possibly due to mass transfer limitations occurring at high solid concentration. Glucan and xylan hydrolysis of 36–52 % and 59–78 %, respectively, were achieved for lower solid concentration (4–6 %) by varying the enzyme loading from 10 FPU/$g_{glucan}$ to 20 FPU/$g_{glucan}$. Despite the lower enzymatic hydrolysis performance at 10 % solid concentration, glucan and xylan hydrolysis yields of 45 % and 63 %, respectively, were achieved by increasing the enzyme loading to 20 FPU/$g_{glucan}$.

For the selection of the enzymatic hydrolysis conditions, ANOVA was used to evaluate the effects of solid concentration and enzyme loading on both sugar concentrations and enzymatic hydrolysis efficiency. Enzyme loading resulted in a significant effect on enzymatic hydrolysis efficiency and glucose concentration ($p \leq 0.05$). Considering that pretreated sawdust still contains a considerable amount of lignin, undesirable and non-productive bindings of enzymes to lignin occur, thereby decreasing enzyme concentration and negatively affecting the enzymatic hydrolysis performance. Furthermore, solid concentration was significant, not for glucan hydrolysis, but for glucose concentration. The conditions for enzymatic hydrolysis that provided maximum glucose release (29.0 g/L) were 10 % solid concentration and 20 FPU/$g_{glucan}$ enzyme loading. These conditions resulted in adequate fermentable sugar concentration (38.5 g/L total sugar) and yield for the continuity of

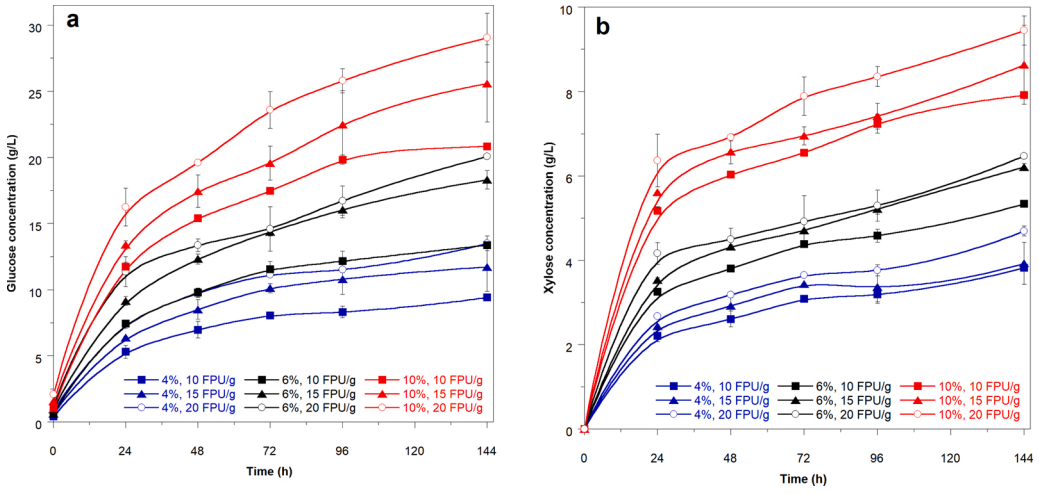


**Fig. 1.** Glucose (a) and xylose (b) concentration profiles during enzymatic hydrolysis of alkaline-pretreated eucalyptus sawdust at different solid concentration (4–10 %) and enzyme loadings (10–20 FPU/$g_{glucan}$).

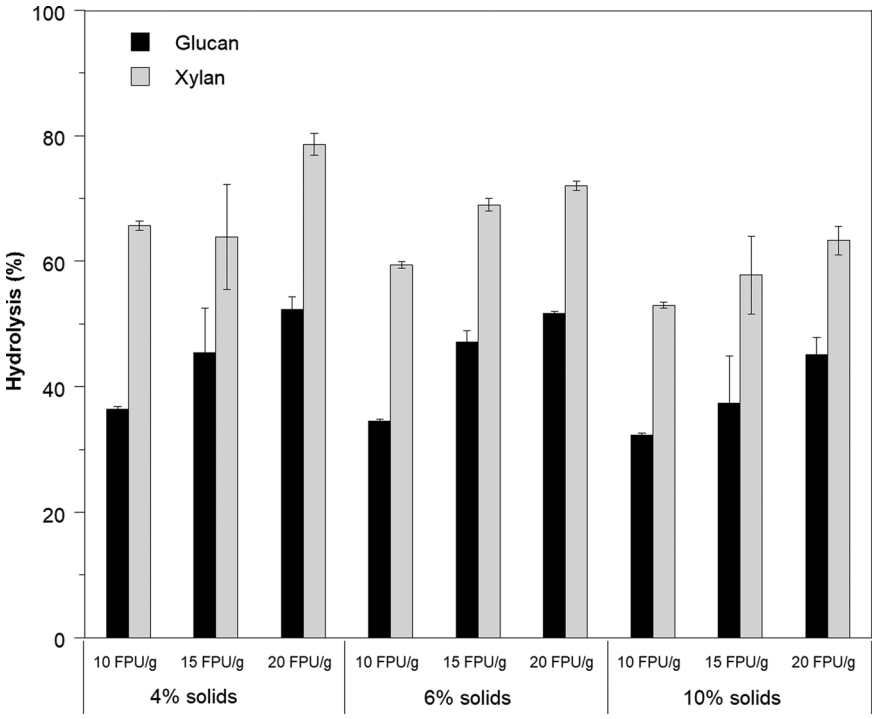


**Fig. 2.** Enzymatic hydrolysis yields for alkaline-pretreated eucalyptus sawdust at different solid concentration (4–10 %) and enzyme loadings (10–20 FPU/$g_{glucan}$).

this work.

### 3.3. Lactic acid batch fermentation from eucalyptus enzymatic hydrolysate

The hydrolysate obtained after enzymatic hydrolysis of alkaline-pretreated eucalyptus, which contained a mixture of glucose and xylose sugars, was used for lactic acid fermentation using *Carnobacterium* sp. under anaerobic conditions at its optimal temperature for growth. Oxygen requirements and optimal growth temperature were previously determined (data not shown), corresponding to the best growth conditions for anaerobiosis at 20 °C, respectively. Although glucose and xylose are two prominent sugars that are present in most lignocellulosic hydrolysates, many microorganisms lack the ability to metabolize both sugars and can only utilize glucose for lactic acid production. However, some LAB, such as *Lactobacillus* spp. and *Leuconostoc* sp., are known to ferment xylose through heterofermentative metabolism, producing lactic acid and byproducts such as acetic acid and/or ethanol [1,33]. In this study, the microorganism employed can metabolize both glucose and xylose as single carbon sources to produce lactic acid (data not shown), with low byproducts production (mainly acetic acid and formic acid, varying according to the fermentation conditions). However, it showed clear preference for the use of glucose. For the batch fermentations, low levels of initial substrate concentration (20 g/L of total sugars) were used to avoid substrate inhibition and allow rapid biomass growth. The initial pH was set at 6.5 and it was monitored during the fermentation process. The fermentation profiles of glucose, xylose, biomass and lactic acid concentration and pH are shown in

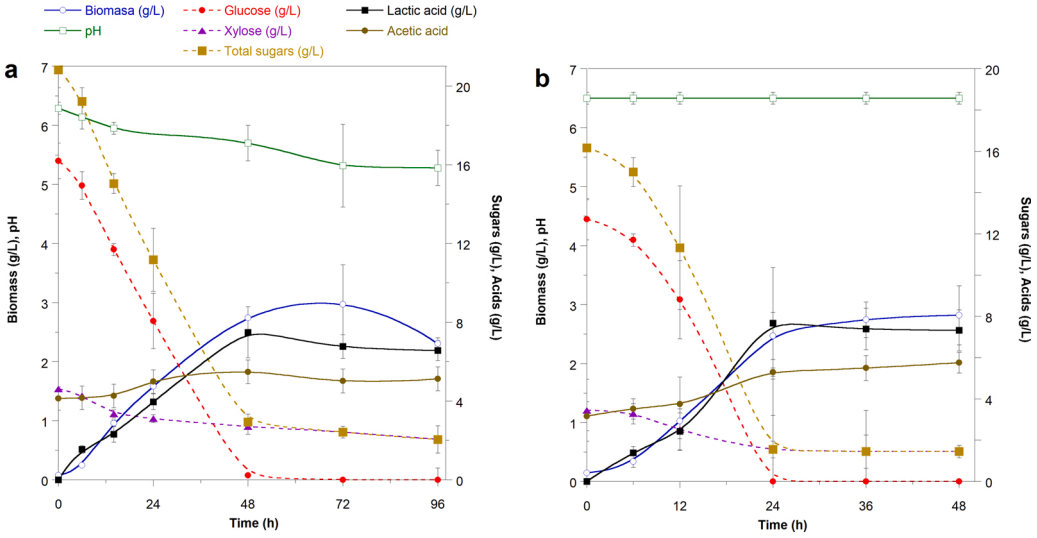


**Fig. 3.** Biomass, lactic acid, acetic acid and sugars concentration and pH profiles during batch fermentation without (a) and with (b) pH control strategy, using the enzymatic hydrolysate produced at 10 % solid concentration and 20 FPU/$g_{glucan}$ enzyme loading.

Fig. 3a. It can be observed that lactic acid production was directly associated with biomass growth, with a maximum lactic acid concentration of 7.4 g/L and biomass concentration of 2.7 g/L achieved after 48 h of fermentation. Glucose concentration decreased sharply and was completely consumed in 48 h, while xylose concentration decreased from 4.6 g/L to 2.7 g/L (41 % conversion). A yield of 42 g of lactic acid per 100 g consumed sugars, a volumetric productivity of 0.16 g/Lh and a fermentation efficiency of 46 % were obtained (Table 2).

Although *Carnobacterium* sp. completely consumed glucose and the process was more rapid than with xylose, both glucose and xylose sugars were simultaneously consumed. It has been reported that the genes required for xylose uptake are repressed in the presence of glucose and that xylose could be utilized when glucose is almost consumed [34]. This relaxed carbon catabolite repression has been previously observed for some LAB [1,35]. For instance, *Lactobacillus* spp. and *Enterococcus* spp. were able to simultaneously ferment mixtures of glucose and other sugars (xylose sucrose and/or fructose) to produce lactic acid from a variety of lignocellulosic materials. The simultaneous utilization of xylose and glucose was also reported by Wang et al. [34] for a *Bacillus sp.* strain for lactic acid production. Grewal and Khare [35] evaluated the lactic acid fermentation by *L. brevis* using a mixture of glucose and xylose sugars as carbon sources and reported complete consumptions of both sugars at the end of the fermentation, although preference for glucose over xylose was observed, producing 13.2 g/L of lactic acid. While glucose conversion was complete, only 41 % conversion was achieved for xylose.

The biomass concentration (2.7 g/L) under the selected conditions was comparable to that reported by de la Torre et al. [36], which reached a concentration of 4−5 g/L using *L. delbrueckii* at 40 °C and pH of 5.8 using an orange peel waste hydrolysate with 110 g/L of initial fermentable sugars. The biomass growth achieved in this part of the study results promising for this psychrotolerant *Carnobacterium sp.*, which was grown in a culture medium using eucalyptus hydrolysate as a carbon source.

Due to the accumulation of lactic acid during batch fermentation, the pH of the fermentation broth decreased from 6.5 to 5.7. Most species of the *Carnobacterium* genus have been reported to grow in a pH range of 6–9 [12,37], so decreasing the pH could inhibit lactic acid fermentation. Moreover, lactic acid is known to be a strong inhibitor of biomass growth and microbial activity in lactic acid fermentation [38]. Therefore, batch fermentation was then performed by controlling the fermentation pH at 6.5 in order to verify whether the pH decrease negatively affected the lactic acid fermentation by *Carnobacterium* sp.

### 3.4. Effect of pH control on lactic acid batch fermentation

Fig. 3b shows the fermentation profiles for the experiment where the pH was controlled at 6.5. During the initial 12 h, similar fermentation performances were observed with and without pH control in the enzymatic hydrolysate, considering that similar lactic acid (2.3–2.4 g/L) and biomass (1.0 g/L) concentrations and sugar conversions (28–38 % glucose and 27–33 % xylose) were reached in both cases. However, from this fermentation time, the lactic acid and biomass productions, as well as the sugar conversions, were influenced by the fermentation pH (Fig. 3b). Although similar lactic acid and biomass concentration, glucose and xylose conversions ($x_{glucose}$ and $x_{xylose}$, respectively), and yield coefficient for biomass production on total sugar consumption ($Y_{X/S}$) were observed at the end of the fermentation (Table 2), greater substrate consumption and lactic acid production rates in the fermentation with pH control enabled the attainment of complete glucose consumption and maximum lactic concentration (7.6 g/L) within 24 h. Thus, the yield coefficient for lactic acid production on total sugar consumption ($Y_{P/S}$) and lactic acid productivity ($Q_P$) were higher with pH control.

When the pH was not controlled at 6.5, the metabolism of *Carnobacterium* sp. was affected since sugar consumption, lactic acid production, and biomass growth slowed down, and the fermentation was extended up to 48 h. It has been reported by some authors that the presence of weak acids (e.g., lactic acid) in the fermentation broth can inhibit bacterial growth because as the external pH decreases, the acid is protonated, resulting in the membrane soluble being able to enter the cytoplasm through simple diffusion [17,39]. As the intracellular pH of most bacteria is close to neutral, the protonated acids inside the cell dissociate and release protons and anions. The accumulation of acid anion inside the cell was reported to inhibit cellular function via several mechanisms. For instance, it has been reported that the accumulation of dissociated acid in the cell can cause growth slowdown and death due to the increase in the cell energy consumption in an attempt to pump out the protons outside the cell [17,26]. Although fermentation ceased at 48 h due to the low levels of substrate concentration used, the results demonstrated that pH control may be necessary when increasing the substrate concentration. This is to avoid cell death due to the low pH levels reached during fermentation, which are ascribed to higher lactic acid production.

Table 2 shows the fermentation parameters obtained in the batch fermentations with and without pH control. Although glucose was completely consumed in both cases, a greater concentration of residual xylose without pH control can be observed, which suggests that xylose conversion (41 %) may be affected by the fermentation pH. When the fermentation pH was controlled at 6.5, the conversion of this pentose (47 %) was enhanced, but a xylose concentration of 1.6 g/L still remained in the fermentation broth at 24 h without further consumption (Fig. 3b).

Along with lactic acid, some LAB can produce byproducts (e.g. acetic acid, formic acid, ethanol) during fermentation, depending on the metabolic pathway used (heterofermentation) [1,9]. For an efficient production of lactic acid, byproduct formation should be avoided or kept at low levels. In this study, low levels of formic acid and acetic acid were detected in the fermentation broth. Formic acid formation was very low, surpassing only 1 g/L in the batch fermentation at controlled pH (1.6 g/L), whereas acetic acid was found in both fermentations as byproduct (1.4 g/L and 2.6 g/L without and with pH control, respectively). This byproduct formation could be due to the fermentation of xylose by the 6-PG/PK pathway. As xylose consumption increased, acetic acid and formic acid production also increased, in accordance to xylose metabolism by the 6-PG/PK pathway. Even though higher levels of acetic acid production were found in this study compared to data reported for other facultative heterofermenters such as *L. plantarum*, no xylose was consumed during *L. plantarum* fermentation in the presence of a mixture of glucose and xylose [40]. Strategies such as high xylose-to-glucose ratios in the culture medium have been proven to cause a metabolic shift from heterofermentation to homofermentation in *Enterococcus mundtii* QU 25 [41]. Moreover, other attempts such as genetic engineering have been carried out through the deletion of genes of the 6-PG/PK pathway or the insertion of the EMP route genes [33,42].

**Table 2**
Stoichiometric parameters for lactic acid (P) production by *Carnobacterium* sp. in different operational modes.

| Parameter | Batch pH free | Batch pH 6.5 | Fed-batch |
|---|---|---|---|
| $t_{fermentation}$ (h) | 48 | 24 | 96 |
| X (g/L) | 2.7 ± 0.7 | 2.5 ± 0.4 | 4.2 ± 0.4 |
| $P_{max}$ (g/L) | 7.5 ± 1.3 | 7.7 ± 1.7 | 29.6 ± 1.5 |
| $Y_{X/S}$ (g/g) | 0.15 ± 0.02 | 0.15 ± 0.02 | 0.07 ± 0.01 |
| $Y_{P/S}$ (g/g) | 0.42 ± 0.05 | 0.50 ± 0.02 | 0.50 ± 0.02 |
| $Q_P$ (g/Lh) | 0.16 ± 0.02 | 0.32 ± 0.02 | 0.31 ± 0.02 |
| *n* (%) | 46 ± 5 | 55 ± 3 | 54 ± 4 |
| $x_{glucose}$ (%) | 100 ± 1 | 100 ± 1 | 100 ± 1 |
| $x_{xylose}$ (%) | 41 ± 6 | 47 ± 5 | 37 ± 5 |

### 3.5. Lactic acid fed-batch fermentation

Although a rapid biomass growth was observed for the batch

fermentation for pH controlled at 6.5, a rapid substrate limitation (of glucose but not xylose) was also observed after 24 h of fermentation due to the low level of initial substrate concentration. In order to increase the production of lactic acid, a pulse feed was added to the fermentation broth when glucose was almost completely consumed (Fig. 4). The total sugar concentration increased up to 59 g/L (45 g/L glucose and 14 g/L xylose). Both lactic acid and biomass concentration increased after the feed pulse, reaching concentrations of 29.6 g/L and 4.2 g/L, respectively. This corresponds quite well with previous reported data, which showed that lactic acid production depends on microbial growth since lactic acid biosynthesis is carried out during the growth phase of the microorganism; thus, the increase in biomass concentration promotes an increase in lactic acid production [17].

Glucose was completely consumed at 96 h fermentation. However, there was still 10 g/L of unconsumed xylose in the culture medium at the end of the fermentation (37 % xylose consumption). The incomplete consumption of xylose by *Carnobacterium* sp. could be due to the exhaustion of other components of the fermentation broth, which may be fundamental for its growth. For instance, it has been reported that high levels of some elements such as $Mn^{2+}$, can improve biomass growth for some *Carnobacterium* spp. [43,44]. Acetic acid production was only observed at the initial batch fermentation (2.3 g/L), while no acetic acid production was observed after the pulse, probably due to the poor consumption of the remaining xylose.

According to the results obtained, the fed-batch fermentation at controlled pH achieved 56 % improvement in biomass growth and 17 % improvement in the lactic acid process efficiency over batch fermentation at uncontrolled pH (Table 2). Similar to the findings in this study, it has also been reported by other authors that lactic acid fermentation at controlled pH and using fed-batch fermentation strategies enhanced lactic acid production. For instance, Romaní et al. [45] reported improvements of 51 %, 5% and 55 % on lactic acid concentration, product yield and productivity by *Lactobacillus rhamnosus* using cellulosic biosludges of *Eucalyptus globulus* in fed-batch fermentation at controlled pH conditions.

Although the lactic acid yield and productivity for fed-batch fermentation were not higher than those of the batch fermentation (0.50 g/g and 0.31 g/L.h, respectively), the final concentration of lactic acid increased from 7.7–29.6 g/L. Moreover, it was demonstrated that L-lactic acid was produced with an optical purity higher than 95 %. A higher product concentration will reduce the product recovery cost. Therefore, the use of a low concentration of initial substrate, followed by the addition of a substrate pulse at the end of the exponential growth, improved the lactic acid production from enzymatic hydrolysate of alkaline-pretreated eucalyptus by *Carnobacterium* sp. Further optimization of media culture components should be performed to improve the strain performance and, thus, lactic acid fermentation yield and efficiency. However, the results in this study showed the biotechnological potential of this wild strain to be use as a lactic acid producer on lignocellulosic materials.

### 3.6. Lactic acid fermentation by different LAB strains

Table 3 compares the results obtained in this work to data reported by other authors on lactic acid fermentation using lignocellulosic substrates and LAB. Several authors have reported data using different *Lactobacillus* sp. strains through both batch and fed-batch fermentation configuration [46,60,61]. However, the lactic acid production by *Carnobacterium* sp. had not been widely studied to date. There are no previous reported studies on lactic acid fermentation by *Carnobacterium*

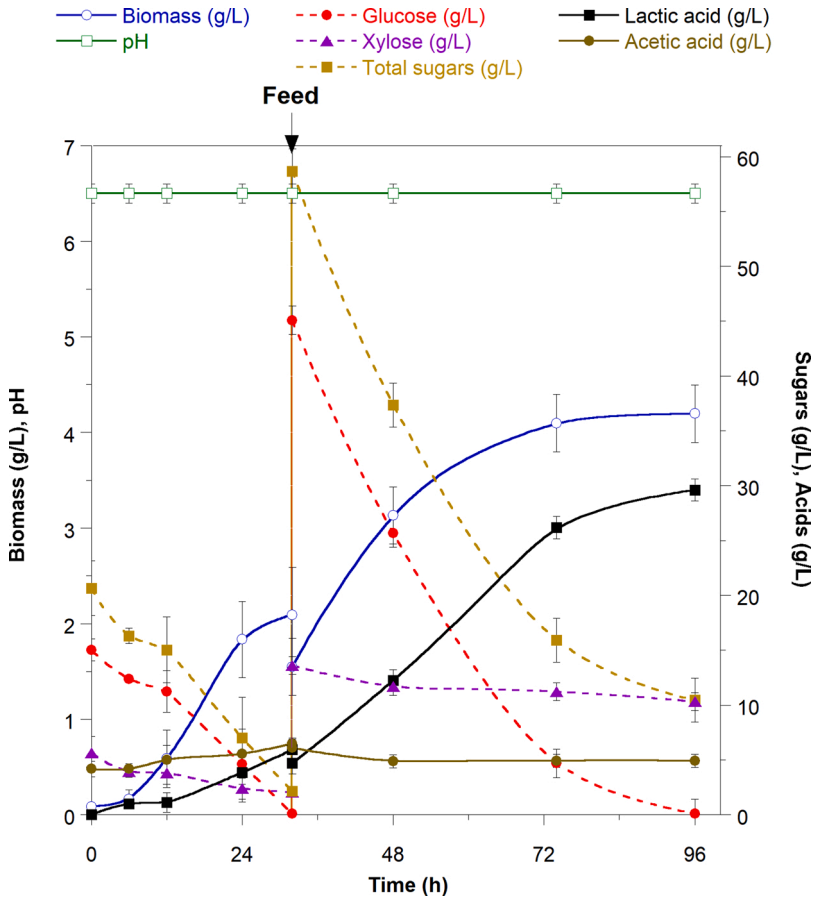


**Fig. 4.** Biomass, lactic acid, acetic acid and sugars concentration and pH profiles during fed-batch fermentation with pH control. The arrow indicates when the substrate feed was added to the fermentation broth (at about 30 h).

**Table 3**
Comparison of literature reported data for lactic acid production from lignocellulosic materials using different lactic acid bacteria (LAB) with results achieved in this work.

| Microorganism | $Y_{P/S}$ (g/g) | Lactic acid (g/L) | Carbon source | Operational conditions | Reference |
|---|---|---|---|---|---|
| *Carnobacterium sp.* | 0.42 | 7.4 | Eucalyptus sawdust hydrolysate | Batch mode, free pH | This work |
| *Carnobacterium sp.* | 0.50 | 7.6 | Eucalyptus sawdust hydrolysate | Batch mode, fixed pH | This work |
| *Carnobacterium sp.* | 0.48 | 29.4 | Eucalyptus sawdust hydrolysate | Fed-batch mode, fixed pH | This work |
| *Lb. brevis* ATCC 367 | 0.39 | 16.3 | Corn stover | SSF, batch mode, fixed pH | Zhang and Vadlani [40] |
| | 0.48 | 18.8 | Poplar hydrolysate | Batch mode, fixed pH | Zhang and Vadlani [40] |
| *Lb. casei* | 0.90 | 48.4 | Lignocellulosic residues of *Sophora flavescens* | SSF, fed-batch mode, free pH | Zheng et al. [48] |
| *Lb. casei* CICC 6056 | 0.83 | 55.1 | *Sophora flavescens* residues | SSF, batch mode, pH adjusted every 12 h | Wang et al. [49] |
| *Lb. coryniformis* | 0.65 | 91.6 | *Curcuma longa* waste | Batch mode, fixed pH | Nguyen et al. [50] |
| *Lb. coryniformis* subsp. *torquens* | 0.97 | 57.0 | Pulp mill residue | SHF, batch mode, fixed pH | de Oliveira et al. [51] |
| *Lb. coryniformis* sp. *torquens* | 0.51 | 23.4 | Waste cardboard | SSF fed-batch, fixed pH | Yáñez et al. [52] |
| *Lb. delbrueckii* subsp. *bulgaricus* MI | NS | 4.74 | Wheat straw hydrolysate | SHF, batch mode, free pH, anaerobic conditions. | Cizeikiene et al. [53] |
| *Lb. delbrueckii* subsp. *bulgaricus* DSM 20,081 | NS | 4.81 | Wheat straw hydrolysate | SHF, batch mode, free pH in anaerobic conditions. | Cizeikiene et al. [53] |
| *Lb. delbrueckii* | 0.83 | 99.8 | Orange peel waste hydrolysate | Batch mode, fixed pH | de la Torre et al. [36] |
| *Lb. paracasei* | 0.69 | 97.1 | *Curcuma longa* waste | Batch mode, fixed pH | Nguyen et al. [50] |
| *Lb. plantarum* ATCC 21,028 | 0.50 | 21.0 | Corn stover | SSF, batch mode, fixed pH | Zhang and Vadlani [40] |
| | 0.87 | 25.6 | Poplar hydrolysate | Batch mode, fixed pH | Zhang and Vadlani [40] |
| *Lb. rhamnosus* | 0.74 | 27.8 | Cellulosic biosludges of *Eucalyptus globulus* | SSF, batch mode, fixed pH [b] | Romaní et al. [45] |
| *Lb. rhamnosus* | 0.78 | 42.0 | Cellulosic biosludges of *Eucalyptus globulus* | SSF, fed-batch mode, fixed pH [b] | Romaní et al. [45] |
| *Lb. rhamnosus* ATCC 7469 | 0.97 | 73.0 | Recycled paper sludge | SSF, batch mode, pH control with $CaCO_3$ | Marques et al. [54] |
| *Lb. sanfranciscensis* MW15 | NS | 4.94 | Wheat straw hydrolysate | SHF, batch mode, free pH | Cizeikiene et al. [53] |
| *Lb. brevis* ATCC 367 and *Lb. plantarum* ATCC 21,028 | 0.80/0.71 | 31.8/28.1 | Poplar hydrolysate | Sequential fermentation/SSF, batch mode, fixed pH | Zhang and Vadlani [40] |
| | 0.78/0.57 | 31.2/24.0 | Corn stover | Sequential fermentation/SSF, batch mode, fixed pH | Zhang and Vadlani [40] |

*Lb.*: *Lactobacillus;* SHF: Separated hydrolysis and fermentation; SSF: Simultaneous saccharification and fermentation; SSCF: Simultaneous Saccharification and co-Fermentation; NS: Not Stated; [a] g lactic acid per gram total solids; [b] aerobic conditions.

strains using lignocellulosic feedstock. Borch and Molin [47] reported L-lactic acid production by two different *Carnobacterium* strains (*C. pisicola* and *C. divergens*) from glucose-based semi-synthetic media and, even though L-lactic acid concentrations were not reported in their work, their product yields (0.21 and 0.28 g/g, respectively) resulted in values that were lower than those achieved in this work (0.42−0.50 g/g).

On the other hand, high lactic acid concentrations (up to 100 g/L) were found in the literature using LAB from *Lactobacillus* spp. from different lignocellulosic materials as carbon sources. These results were obtained for D-lactic acid or racemic mixtures of D- and L-lactic acid production [36,50]. Nguyen et al. [50] reported concentrations of 91.6 g/L and 97.1 g/L racemic mixtures of D- and L-lactic acid for batch fermentations by *L. coryniformis* and *L. paracasei*, respectively, from *Curcuma longa* hydrolysate at controlled pH and anaerobic conditions, reaching 0.65−0.69 g/g product yields and 2.1–2.7 g/L.h productivities. The production of racemic mixtures presents downstream disadvantages considering that the isomers separation is laborious, thereby increasing process costs and decreasing production yields. In addition, obtaining the pure L-isomer is beneficial since it is suitable for human consumption and, thus, useful for food applications.

L-Lactic acid production was also investigated from different lignocellulosic resources using mostly *Lactobacillus* spp., achieving concentrations of the pure L-isomer in the range of 14−48 g/L [45,48,55]. Additionally, it was previously reported that *Carnobacterium* spp. can produce pure L-lactic acid from both hexoses and pentoses [56,57]. The *Carnobacterium* sp. strain used in this study achieved comparable L-lactic acid concentrations and yields to others L-lactic acid producers' bacteria from the *Lactobacillus* genus using similar types of lignocellulosic raw materials. Moreover, its improved performance in fed-batch fermentation configuration demonstrated its potential as an alternative lactic acid producer from lignocellulosic resources.

Besides LAB, some bacterial genera not belonging to LAB and certain fungi have also been reported to produce lactic acid from different carbon sources, including lignocellulosic substrates. For instance, *Bacillus* spp. such as *B. coagulans* and *B. subtilis* strains have recently shown potential for lactic acid production due to its capacity to metabolize pentose sugars and produce optically pure L-lactic acid with high yields [19,46,58,59]. Among fungi, some *Rhizopus* sp. strains have been reported to produce high lactic acid concentration. However, they produce other by-products unlike homofermentative LABs. Genetic modification of wild type microorganisms has improved their lactic acid production [46].

## 4. Conclusions

Lactic acid fermentation by *Carnobacterium* sp., a LAB isolated from a maritime Antarctic lake, was investigated using eucalyptus sawdust as renewable feedstock. Glucose and xylose, which are fermentable sugars extracted from alkaline-pretreated eucalyptus sawdust through enzymatic hydrolysis, were successfully utilized as carbon sources by *Carnobacterium* sp. Although *Carnobacterium* sp. consumed both glucose and xylose, a preference for glucose over xylose was observed during fermentation. Moreover, complete xylose consumptions were not

achieved. Fed-batch fermentation and pH control strategy increased both lactic acid (30 g/L) and biomass concentration (4.2 g/L) compared to the values obtained for batch fermentations. Through this strategy, a product yield of 50 g lactic acid per 100 g of sugars was achieved, along with a volumetric productivity of 0.31 g/Lh and L-lactic acid optical purity higher than 95 %. These results demonstrated the potential of using eucalyptus sawdust for cellulosic L-lactic acid production by *Carnobacterium* sp. Under the conditions evaluated in this study, the proposed process allowed to obtain 114 g of lactic acid per 1 kg of eucalyptus sawdust. Future research should focus on identifying effective, low-cost nutritional supplements that would enable the attainment of complete substrate conversion and high lactic acid production yields.

## CRediT authorship contribution statement

**Laura Camesasca:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization, Project administration. **Juan Andrés de Mattos:** Conceptualization, Methodology, Validation, Formal analysis, Investigation. **Eugenia Vila:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization. **Florencia Cebreiros:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization. **Claudia Lareo:** Writing - review & editing, Supervision.

## 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.

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