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Valorisation of fungal hydrolysates of exhausted sugar beet pulp for lactic acid production.

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Abstract

BACKGROUND: Exhausted sugar beet pulp pellets (ESBPP) were used as raw material for lactic acid (LA) fermentation. The enzymatic hydrolysis of ESBPP was performed with the solid obtained after the fungal solid-state fermentation of ESBPP as a source of hydrolytic enzymes. Subsequently, a medium rich in glucose and arabinose was obtained, which was used to produce LA by fermentation. For LA production, two *Lactobacillus* strains were assayed and the effect of the supplementation of the hydrolysate with a nitrogen source and the mode of pH regulation of the fermentation were investigated. Moreover, a kinetic model for lactic acid fermentation by *Lactobacillus plantarum* on ESBPP hydrolysates was developed.

RESULTS: *L. plantarum* produced a LA concentration 34 % higher than *Lactobacillus casei*. The highest LA concentration (30 g L^{-1}) was obtained with *L. plantarum* when the hydrolysate was supplemented with 5 g L^{-1} of yeast extract and the pH was controlled

with $CaCO_3$. The concentration of acetic acid differed depending on the concentration of $CaCO_3$ added, producing its maximum value with 27 g L^{-1} of $CaCO_3$. The proposed kinetic model was able to predict the evolution of substrates and products depending on the variation of the pH in the hydrolysate, according to the amount of $CaCO_3$ added.

CONCLUSION: ESBPP can be revalorized to produce lactic acid. A pure LA stream or a mixture of lactic and acetic acid, depending on the pH control method of the fermentation, can be produced. Thus, this control shows a great interest depending on the destination of the effluent.

Key words

Lignocellulosic biomass, Lactic acid fermentation, *Lactobacillus*, pH control, kinetic model, nitrogen supplementation

1 Introduction

Lactic acid bacteria (LAB) are used to produce a wide variety of chemicals with high commercial interest, being lactic acid (LA) the most predominant industrial product. Lactic acid is a versatile organic acid that is widely used in the food industry as a preservative (acidifier) and flavour-enhancing agent[1-3]. Also, lactic acid bacteria are used in food industry due to their functional activity, as starters and potential probiotic strains in preparation of dairy products[4,5]. LA is also extensively employed in cosmetics formulations owing to its emulsifying and moisturizing effects on the skin. In the pharmaceutical industry, it is used for the synthesis of dermatologic products and

against-osteoporosis drugs[6]. In addition to these uses, LA has gained interest as a precursor of polylactic acid (PLA), which is a bio-degradable and bio-based bio-plastic[7].

The production of lactic acid can be performed by chemical or fermentative pathways, being the last one more environmentally friendly. One advantage of the fermentative process is that it gets the chance to use low-cost raw materials, like lignocellulosic biomass, as nutrient sources for microbial growth. These substrates have the additional advantage that they do not compete with food-crops[8].

Lignocellulosic biomass is the main component of cell walls in plants. It is mainly composed by lignin and three polysaccharides: cellulose, hemicellulose, and pectin[9]. Cellulose and hemicellulose fractions are polymers made up of monosaccharides and, therefore, they constitute a potential source of fermentable sugars, which could be converted into high added-value products.

The common steps involved in the fermentative pathway to produce LA from lignocellulosic materials are pretreatment, enzymatic hydrolysis, fermentation, separation and purification[10]. Fermentation is usually carried out by *Lactobacillus* species[11]. During this process, the lactic acid yield is influenced by several experimental conditions, including temperature, pH, carbon source, initial sugar concentration, aeration rate, agitation speed, medium composition, inoculum size and age and fermentation mode (continuous, semi-continuous or batch fermentations).

In this study exhausted sugar beet pulp pellets (ESBPP) are used as raw material for lactic acid fermentation. Sugar beet pulp is a sub-product obtained after the diffusion step in the sugar production process from sugar beet. It is normally dehydrated, pelletized and

sold for animal feeding. The ESBPP used in this study are mainly composed of pectin (0.41 g g^{-1}), cellulose (0.25 g g^{-1}), hemicellulose (0.16g g^{-1}) and lignin (0.03g g^{-1})[12]. ESBPP also contain a low amount of lignin, and therefore do not require a harsh pretreatment before the enzymatic hydrolysis[13]. In order to develop a low-cost process, the cost of the hydrolysis step was reduced by also using ESBPP as solid substrate to produce hemicelluloses by *Aspergillus awamori* fermentation. In this way, the fermented ESBPP, containing mainly hemicelluloses, was supplemented with a commercial source of cellulases and were used for the hydrolysis of fresh ESBPP. A flowchart of the process can be found in Figure 1.

For lactic acid fermentation, *Lactobacillus plantarum* and *Lactobacillus casei*, two common lactic acid bacteria (LAB) used to produce lactic acid were tested[14–17]. Two different studies were carried out: a) the influence of the supplementation of the hydrolysates with a nitrogen source; and b) the influence of the regulation of pH during the process.

Finally, a kinetic model for the production of lactic and acetic acids was developed, taking into account the concentration of glucose and arabinose in the hydrolysates and the effect of pH regulation. Kinetic models of the fermentation processes are a very useful tool at industrial scale, because they help to find the optimal conditions by performing only a few experimental tests[18]. In addition, they are also very useful for the interpretation of the production results obtained in industrial plants.

2 Material and methods

2.1 Raw material

ESBPP were used as raw material to produce the hydrolysates rich in simple sugars. They were supplied by the sugar plant Azucarera del Guadalete (AB Sugar Group), located in Jerez de la Frontera (South of Spain). Samples were collected and stored at 4 °C until use. As it has been mentioned, the total carbohydrate polymers content of ESBPP is around 0.83 g g^{-1} of total weight[12].

2.2 Production of ESBPP hydrolysates

The culture media used for lactic fermentations were obtained from the enzymatic hydrolysis of ESBPP[19].

2.2.1 Strain and inoculum growth

The fungus *Aspergillus awamori* 2B.361 U2/1, a sequential mutant of *Aspergillus niger* NRRL 3312, was used in this study. The strain spores were stored for their maintenance in glycerol (0.50 mL mL^{-1}) at −25 °C. The fungus was propagated by spreading 0.1 mL of the spore solution onto Petri dishes containing a synthetic medium composed by (g L^{-1}): 1 peptone, 0.5 yeast extract, 15 agar, 6 xylan, 5 avicel and 1 pectin. The Petri dishes were incubated at 30 °C for 5 days. After the incubation period, the spores were collected by adding a 9 g L^{-1} NaCl solution to the plates, followed by gentle scraping. The number of spores in the suspension was counted using an improved Neubauer chamber.

2.2.2 Raw material conditioning

ESBPP were soaked in distilled water (3 g of solid in 0.1 L of water) to disrupt the pellet conformation and then dried in an oven at 40 °C for 24 h. After that, ESBPP were sterilized by autoclaving at 120 °C for 20 min.

2.2.3 Solid state fermentation

Solid-state fermentations were performed by adding to disposable Petri dishes 5 g of dried and sterilized ESBPP, the volume of spores suspension required to obtain a final inoculum concentration of $1 \cdot 10^7$ spores g^{-1} of solid and the appropriate amount of a nutrient solution to adjust the initial moisture content to 70 % . The nutrient solution composition was as follows (g L^{-1}): 2.4 urea, 9.8 $(NH_4)_2SO_4$, 5.0 KH_2PO_4, 0.001 $FeSO_4 \cdot 7H_2O$, 0.0008 $ZnSO_4 \cdot 7H_2O$, 0.004 $MgSO_4 \cdot 7H_2O$ and 0.001 $CuSO_4 \cdot 5H_2O$ at pH 5.0. pH was not controlled and the plates were incubated under static conditions at 30 °C for 8 days[12].

2.2.4 Enzymatic hydrolysis

Enzymatic hydrolysis was carried out by directly adding 15 g of fermented ESBPP, as a source of hydrolytic enzymes, to 55 g of sterilized ESBPP in 0.3 L of citrate-phosphate buffer (pH 5, 0.05 M). This mixture was supplemented with 2.17 units of cellulases from the commercial preparation Celluclast® per gram of fresh ESBPP. The enzyme activities of the fermented ESBPP were measured in a previous published work[19], which were 24.6 U of xylanase per 1 g of dried fresh ESBPP, 9.3 U of exo-polygalacturonase per 1 g of dried fresh ESBPP, and 1.9 U of cellulase per 1 g of dried fresh ESBPP, being β-

glucosidase activity null[19]. Therefore, as cellulase activity was low, Celluclast® was added to the fermented solid. The process was carried out in batch mode in Erlenmeyer flaks (500 mL). The solid suspensions were continuously mixed by incubating the flaks in an orbital shaker at 200 rpm and 50 ºC for 5 days. After that, the whole content of the Erlenmeyer flaks was autoclaved (120 ºC – 20 min) and frozen until later use.

2.3 Lactic acid fermentation

2.3.1 Strains maintenance and inoculum preparation

Lactobacillus plantarum (CECT 748) and *Lactobacillus casei* (2246 from the strains collection of the University of Parma) were used individually for lactic acid fermentations. Both bacterial strains were maintained as frozen stocks (−70 ºC) in Man Rogosa Sharpe (MRS) medium, supplemented with glycerol (0.15mL mL^{-1}). MRS medium was composed by (g L^{-1}): 15 glucose, 10 peptone, 10 meat extract, 5 yeast extract, 5 sodium acetate, 2 ammonium citrate, 0.2 $MgSO_4 \cdot 7H_2O$, 0.05 $MnSO_4 \cdot 7H_2O$ and 2 K_2HPO_4.

For inoculum preparation, 6 mL of MRS medium were inoculated with 100 µL of the bacterial frozen stocks and incubated in anaerobic conditions at 30 ºC for 24 h. Afterwards, the cultures were propagated two more times in 6 mL of MRS medium with 200 µL of the previous culture at the same conditions.

2.3.2 Lactic acid fermentation in synthetic medium

The growth of *Lactobacillus plantarum* was tested in three MRS mediums with different carbon sources (glucose, xylose or arabinose). The same test was performed on *L. casei*

in a previously published work[20]. Fermentations were carried out in 250 mL Erlenmeyer flaks by adding 50 mL of the synthetic medium supplemented with the neutralising agent $CaCO_3$ (9 g L^{-1}) and 0.5 mL of inoculum (10^9 cell ml^{-1} aprox.). The synthetic medium used was composed by (g L^{-1}): 15 sugar (glucose, xylose or arabinose), 10 peptone, 10 meat extract, 5 yeast extract, 5 sodium acetate, 2 ammonium citrate, 0.2 $MgSO_4 \cdot 7H_2O$, 0.05 $MnSO_4 \cdot 7H_2O$ and 2 K_2HPO_4. The flasks were closed with cotton plugs and incubated at 30 °C and 150 rpm for 5 days. Samples were collected every 24 h throughout the fermentation and stored at −25 °C for later analysis. Fermentations were carried out in triplicate.

2.3.3 Lactic acid fermentation in ESBPP hydrolysates

Lactic acid fermentations were carried out by adding 0.01 mL mL^{-1} of inoculum to 50 mL of ESBPP hydrolysate in 250 mL Erlenmeyer flaks. The flaks were closed with cotton plugs and incubated at 30 °C and 150 rpm for 5 days. Samples were collected throughout the fermentation and stored at −25 °C for analysis. Fermentations were carried out in triplicate.

For studies of supplementation with different nitrogen sources, 5 g L^{-1} of yeast extract or peptone were added to ESBPP hydrolysates. This study was performed with *L. plantarum* and *L. casei*.

To study the effect of pH regulation on LA fermentation by *L. plantarum*, different concentrations of $CaCO_3$, as neutralising agent, were tested: 9, 18, 27, 55 and 75 g L^{-1}. Moreover, another experiment was carried out maintaining the pH constant at 6.5 by

adding manually a 5 M NaOH solution. For those two studies, ESBPP hydrolysates were supplemented with 5 g L^{-1} yeast extract.

Four different yields were calculated, being two of them calculated at the maximum lactic acid concentration and the other two at the maximum acetic acid concentration. Thus, maximum lactic acid yield ($Y_{Lmax/S,}$ g g^{-1}) was calculated by dividing the maximum lactic acid concentration measured in the medium by the theoretical concentration of lactic acid that could be obtained from the initial sugars (glucose and arabinose). Lactic acid yield ($Y_{L/S,}$ g g^{-1}) was calculated by dividing the lactic acid concentration obtained at the instant of the maximum acetic acid concentration by the theoretical concentration of lactic acid that could be obtained from the initial sugars. Maximum acetic acid yield ($Y_{Cmax/S,}$ g g^{-1}) was calculated by dividing the maximum acetic acid concentration in the medium by the theoretical concentration of acetic acid that could be obtained from the initial sugars. Acetic acid yield ($Y_{C/S,}$ g g^{-1}) was calculated by dividing the acetic acid concentration obtained at the instant of the maximum lactic acid concentration by the theoretical concentration of acetic acid that could be obtained from the initial sugars.

2.4 Sample analysis

Samples were collected during lactic acid fermentation every 24 h. For acids and sugars analysis, samples were centrifuged at 10,621 xg for 10 min, collecting the supernatant and discarding the precipitate. Reducing sugars (RS) in supernatants were analysed by the dinitrosalicylic acid method (DNS) adapted to microplate[21,22]. Glucose concentration was measured by using enzymatic assay kit from Biosystems (D-Glucose/D-Fructose).

Arabinose plus galactose were measured with the enzymatic assay kit from Megazyme (L-Arabinose/D-Galactose assay kit), using arabinose as standard.

Lactic and acetic acids were measured by ionic chromatography (Metrohm, 930 Compact IC Flex, Switzerland) with conductivity detection and Metrosep Organic Acids - 250/7.8 column (Metrohm). The separation was carried out using as eluent a solution composed by 0.4 mmol L^{-1} sulfuric acid and 0.12 mL mL^{-1} acetone, at an isocratic flow rate of 0.4 mL min^{-1}.

The cell growth on LA fermentation was measured using the colony forming unit (CFU) counting method. Samples taken during fermentations were serially diluted in NaCl solution (9 g L^{-1}) and cultured in MRS-agar plates. The plates were incubated in a culture oven at 37 °C for 48 h.

2.5 Statistical analysis

All experiments and assays were performed in triplicate. Statgraphics 18 was used for data analysis. Data were analysed using one-way analysis of variance (one-way ANOVA) and Fisher's least significant differences (LSD, $P < 0.05$) was used to determine significant differences among tested conditions.

2.6 Kinetic model for lactic acid fermentation

In this work, a specific kinetic model for the lactic acid fermentation of *Lactobacillus plantarum* on ESBPP hydrolysates has been developed, which is based on the experimental results presented. The model considers that, in the experimental

conditions involved, *Lactobacillus plantarum* metabolises glucose (eq. 1), arabinose (eq. 2) and lactic acid (eq. 3), for the biomass growth, according to the following three global metabolic reactions:

(1) $\underset{glucose}{C_6H_{12}O_6} \rightarrow \underset{lactic\ acid}{2\ C_3H_6O_3}$

(2) $\underset{arabinose}{C_5H_{10}O_5} \rightarrow \underset{lactic\ acid}{C_3H_6O_3} + \underset{acetic\ acid}{C_2H_4O_2}$

(3) $\underset{lactic\ acid}{C_3H_6O_3} + O_2 \rightarrow \underset{acetic\ acid}{C_2H_4O_2} + CO_2 + H_2O$

The model also considers that the rate of total biomass production depends additively on the consumption of these three carbon sources (glucose, arabinose and lactic acid) (eq. 4). So, when there is more than one carbon source in the medium, the bacterial growth is calculated with the sum of the growth from each of these sources. Moreover, it has been considered that the bacteria growth follows the general Monod equation (eq. 5), with particular parameters in each case (μ_o and K_M).

(4) $$\frac{dX}{dt} = \left(\frac{dX}{dt}\right)_1 + \left(\frac{dX}{dt}\right)_2 + \left(\frac{dX}{dt}\right)_3 \quad ; \quad \left(\frac{dX}{dt}\right)_1 = \mu_G\, X_v \quad ; \quad \left(\frac{dX}{dt}\right)_2 = \mu_A\, X_v \quad ; \quad \left(\frac{dX}{dt}\right)_3 = \mu_L\, X_v$$

(5) $$\mu_G = \mu_{Go}\frac{G}{K_{MG} + G} \quad ; \quad \mu_A = \mu_{Ao}\frac{A}{K_{MA} + A} \quad ; \quad \mu_L = \mu_{Lo}\frac{L}{K_{ML} + L}$$

Here, X represents the total biomass concentration (cell L^{-1}) and G, A and L represent the glucose, arabinose and the lactic acid concentrations in the medium (mol L^{-1}) respectively, at time t (h). The three μ_o coefficients are the maximum specific growth rates of the involved bacteria for each carbon source (cell L^{-1} h^{-1}). Finally, the three K_M coefficients are the corresponding saturation constant for the growth of this bacteria on each carbon source (mol L^{-1}), according to Monod equation.

On the other hand, it has been also considered that biomass viability is reduced, due to the presence of acid cations in the medium, which are produced during the fermentation. Specifically, these cations are the hydrogen ions which come from the produced organic acids (lactic or acetic acid). As a result, hydrogen ions are accumulated into the cell cytoplasm as fermentation proceeds and acids are generated, producing the cell death. Then, it has been considered that the biomass death rate has a linear dependence on the concentration of acids in the medium, following the Chick's Law of disinfection (eq. 6), where subscript *d* represents biomass death. Here, μ_d is the specific death rate (cell L^{-1} h^{-1}) of the involved bacterium, caused by these acids, k_d is the Chick disinfection coefficient for these acids (cell mol^{-1} h^{-1}), X_v represents the viable biomass concentration (CFU L^{-1}), C the acetic acid concentration, and L the lactic acid concentration (both in mol/L).

$$\left(-\frac{dX_v}{dt}\right)_d = \mu_d \cdot X_v \quad ; \quad \mu_d = k_d\,(L + C) \tag{6}$$

Therefore, the evolution of viable biomass through the fermentation is the result of both phenomena: the biomass growth and the viable biomass death (eq.7):

$$\frac{dX_v}{dt} = \frac{dX}{dt} - \left(-\frac{dX_v}{dt}\right)_d \tag{7}$$

In relation to the substrate consumption, it has been considered that it is directly a growth associated process (eq. 8). Thus, consumption rates of the three considered substrates (glucose, arabinose and lactic acid) are proportional to growth rate, as follows:

(8) $\left(-\frac{dG}{dt}\right) = Y_{G/X}\left(\frac{dX}{dt}\right)_1$; $\left(-\frac{dA}{dt}\right) = Y_{A/X}\left(\frac{dX}{dt}\right)_2$; $\left(-\frac{dL}{dt}\right)_3 = Y_{L/X}\left(\frac{dX}{dt}\right)_3$

Where $Y_{G/X}$, $Y_{A/X}$ and $Y_{L/X}$ are respectively the yields of biomass from glucose, arabinose and lactic acid.

Finally, considering the stoichiometric coefficients for reactions 1, 2 and 3, the following product formation rates (lactic and acetic acids) are defined (eq. 9 and 10):

(9) $\frac{dL}{dt} = \left(\frac{dL}{dt}\right)_1 + \left(\frac{dL}{dt}\right)_2 - \left(-\frac{dL}{dt}\right)_3$; $\frac{dL}{dt} = 2\,Y_{L/G}\left(-\frac{dG}{dt}\right) + \left(-\frac{dA}{dt}\right) - \left(-\frac{dL}{dt}\right)_3$

(10) $\frac{dC}{dt} = \left(\frac{dC}{dt}\right)_2 + \left(\frac{dC}{dt}\right)_3 = \left(-\frac{dA}{dt}\right) + \left(-\frac{dL}{dt}\right)_3$

Given that portion of the total glucose present in the medium is assimilated by the biomass to synthesize most components of the new cells, this glucose does not follow reaction 1. As a consequence, the quantity of lactic acid formed by reaction 1 is only the fraction $Y_{L/G}$ of the total glucose consumed. However, the quantity of lactic acid formed by reaction 2 is assumed to be the stoichiometrically corresponding to the total arabinose consumed.

As it can be seen, the proposed kinetic model contains actually only five differential equations: one for each process variable to be calculated (X_v, G, A, L and C), which can be combined and condensed into five. Also, there are only eleven kinetic parameters to be set: three growth parameters (μ_{Io}, K_{MI}, and $Y_{I/X}$) on the three carbon sources (glucose, arabinose and lactic acid), one parameter for the Chick's constant (k_d), and the glucose assimilation coefficient ($1-Y_{L/G}$). Initially, it might seem that there are many parameters to be calculated in the proposed model, however, most of them can be taken directly

from the literature. Some of them are very common fermentative parameters, and others can be calculated in simple separate experiments (no fermentative ones). Obviously, if mass concentrations are used instead of molar concentrations for the compound's variables, their molecular weights must be introduced into the equations. Moreover, mass/cell coefficients must be introduced if biomass concentration is used in cell mass instead of cells number.

Despite the evolution of the principal compounds can be calculated from the above-mentioned equations, pH evolution in the fermentative medium must be calculated separately. Regarding to the presence of hydrogen cations in the medium, two cases can be distinguished: a) regular LA fermentation; and b) LA fermentation with pH control.

In the case of a regular fermentation, it is supposed that hydrogen cations in the medium *(H)* come from the dissociation of the weak acids: lactic acid (L) and acetic acid (C). Therefore, H can be calculated from the classical dissociation equation for weak acids (eq. 11):

(11) $$H = \sqrt{K_a \cdot a} \quad ; \quad H = \sqrt{K_L \cdot L} + \sqrt{K_C \cdot C}$$

Where K_a is the dissociation constant of the weak acid and "a" the acid concentration. Thus, in this case, bearing in mind that the principal acids in the medium are acetic and lactic acids, this general equation can be directly applied, taking for the constants the following values: $pK_L = 3.86$ and $pK_C = 4.80$.

In the case of an LA fermentation with pH control, it is supposed that the formed acids first neutralize the alkali that has been initially added; and, once all the alkali has been

neutralized, the concentration of the hydrogen cations in the medium begins to increase. Thus, it should be applied the general theory of acid–base titrations in order to calculate the resultant pH in the medium in each instant. For this purpose, it can be defined a titration factor (f), at any time of fermentation, as follow:

$$(12) \quad f = \frac{L + C}{B} \quad ; \quad 0 \leq f \leq 1 \Rightarrow H = \sqrt{K_w} \quad ; \quad \forall f > 1 \Rightarrow H = \sqrt{\overline{K}_a \cdot (L + C - B)}$$

Where B is the initial alkali concentration added (mol L^{-1}), in terms of acid–base equivalents; and K_w is the water dissociation constant (10^{-14} M). Before the total alkali neutralization is achieved ($0 \leq f \leq 1$), the hydrogen concentration is only related with the water dissociation. After that point ($\forall f > 1$), the hydrogen concentration is related mainly to the acid's dissociation. For example, if calcium carbonate is used as the neutralizing agent, the following dissociation reaction must be considered for this alkali:

$$(13) \quad CaCO_3 + H_2O \rightarrow Ca^{2+} + 2\,OH^- + CO_2$$

Thus, here B is twice the initial alkali concentration added to the medium. Also, in eq. 12, $\overline{K}_a$ is the overall dissociation constant for the present free acids. Due to these acids are mainly lactic and acetic acids, this overall constant must be calculated here as the average of their dissociation constants. Finally, the sum [$L+C-B$] represents the not-neutralized acid concentration in the medium. Essentially, this second case with pH control is analogous to the first one without this control, but it considers the previous neutralizing effect exerted by the added alkali.

To solve the differential equations system of this model, numerical methods must be applied. Specifically, it has been used the numerical method RK4, a variant of the Runge–

Kutta one[21], due to its better convergence properties. In order to implement the method, specific computer routines have been developed with the program MatLab-Simulink (Mathworks Inc., USA).

This numerical procedure offers the theoretical evolution of seven process variables: X, Xv, G, A, L, C and pH, starting at the at the experimental initial values (boundary conditions) and going through the time until the end of the fermentation (zero viable biomass concentration). The values of these parameters have been fitted using a set of experimental data, both of regular LA fermentations and with pH control. For this purpose, it has been followed the damped least squares analysis[22], being as high as possible the non-linear regression coefficient of all the calculated curves for all the experimental data (R^2) .

3 Results and discussion

3.1 Sugar consumption in synthetic media

One of the two bacteria employed in this article to produce lactic acid from ESBPP hydrolysate is *Lactobacillus plantarum* (CECT 478). This hydrolysate was composed by approximately 64.3 g L^{-1} of total reducing sugars, being 29.1 g L^{-1} of glucose, 21.8 g L^{-1} of arabinose and the rest a mixture of other minority sugars, mainly xylose. Due to the hydrolysate composition and the fact that this strain has been classified as facultative heterofermentative, which means that can utilize hexoses and pentoses[25], *Lactobacillus plantarum* was grown in three synthetic mediums with glucose, arabinose or xylose to identify which sugars were able to be metabolized.

As it can be observed in Figure 2, glucose and arabinose are almost consumed in the synthetic medium, while xylose is not consumed. These results agree with Zhang et al., where it is mentioned that *L. plantarum* is not able to metabolize xylose[26].

The other bacterium used in this work was *Lactobacillus casei* (2246), which is also considered to be a facultative heterofermentative one. This strain was tested in a previous study obtaining, like for the other strain under study, that it could metabolize only glucose and arabinose among all the sugars tested[20].

3.2 Lactic fermentation in ESBPP hydrolysate

Preliminary experiments of lactic acid fermentation in ESBPP hydrolysate were performed with *Lactobacillus plantarum* and *Lactobacillus casei*. These fermentations were carried out without nutrients supplementation and with no pH control.

Different results were obtained for both strains. Thus, *L. plantarum* achieved a maximum lactic acid concentration (L_{max}) of 6.55 ± 0.09 g L^{-1}, after 120 h of fermentation, and a maximum acetic acid concentration (C_{max}) of 0.79 ± 0.20 g L^{-1}, after 72 h. However, for *L. casei*, the value of L_{max} was 4.34 ± 0.26 g L^{-1}, after 120 h of fermentation, and the value of C_{max} was 1.80 ± 0.04 g L^{-1}, after 168 h. Thereby, at the conditions tested, *L. plantarum* produced a higher concentration of lactic acid than *L. casei*, from the same amount of initial sugars. Specifically, the increase in the lactic acid production was around 34 % for the *L. plantarum* strain over the *L. casei* strain.

Considering that the initial reducing sugars concentration in these experiments was 64.3 g L^{-1}, and the initial concentrations of glucose and arabinose were 30 and 22 g L^{-1}

respectively, the theoretical concentration of lactic acid that would be produced was 43 g L^{-1}. So, the concentration of lactic acid obtained at these conditions was around the 10 % of the theoretical production. These results might be explained based on the lack of nutrients in the ESBPP hydrolysate. In literature, there are some authors who supplement the sugars hydrolysate with the usual components of MRS medium, while others only add a nitrogen source or vitamins. Mussatto *et al.* carried out the lactic fermentation with *Lactobacillus delbrueckii*, supplementing the hydrolysate of pretreated brewer's spent grain with 5 g L^{-1} of either yeast extract or the nutrients of MRS medium, obtaining better yields in the last case[11]. In the same way, Zhang and Vadlani *et al.* performed a simultaneous saccharification and fermentation with *L. delbrueckii* of pulp or corn stover and supplemented the medium with the nutrients of MRS medium[27]. Finally, Nancib *et al.* performed several fermentations with *L. casei subps. rhamnosus* on data juice supplemented with different substances (5 vitamins sources and 6 nitrogen sources), obtaining the highest yield with the addition of yeast extract[28]. Thus, next fermentations were carried out using a nutrient supplement for the ESBPP hydrolysate, as it can be seen below.

Regarding pH values measured during the fermentation of both strains, it was observed a decrease from 6.00 to 4.89, at 48 h of fermentation. This effect is produced by the increment of lactic and acetic acid concentrations in the medium. The optimum pH value for the LAB growth is between 5 and 7[29]. Although they are able to tolerate acid conditions, if they are exposed to low pH for a long time, their physiology and metabolism can be affected[6]. Thus, due to the observed pH drop, two different pH regulations methods for the fermentations of ESBPP were compared as it can be seen

below. Other authors have also used different strategies to regulate the pH during the fermentative processes. In this way, Penjin *et al.* used 20 g L^{-1} $CaCO_3$ to regulate the pH in the lactic fermentation on brewer's spent grain, with *Lactobacillus fermentum* and *Lactobacillus rhamnosus.* Hetényi *et al.* studied the effect of five pH regulating methods, obtaining better results with trimethylamine[29].

3.2.1 Effect of nitrogen source supplementation

After the preliminary experiments exposed above, the hydrolysate medium was supplemented with 5 g L^{-1} of either yeast extract or peptone, which are commonly used as nitrogen sources to supplement sugar hydrolysates. Regarding *L. plantarum* and *L. casei* growth, similar trends can be observed on the three media studied (no supplement, yeast extract supplement and peptone supplement), increasing cell counting from 10^7 to 10^{11} cell L^{-1} after 120 h of fermentation in all cases.

The evolution of lactic acid concentration produced by *L. plantarum* is shown in Figure 3A. The maximum lactic acid concentration (L_{max}) was achieved after 120h of fermentation with both nitrogen sources, being 9.36±0.80 g L^{-1} in the medium supplemented with yeast extract and 8.85±0.64 g L^{-1} in the medium with peptone. No significant differences were obtained between both nitrogen sources employed (p-value > 0.05). However, comparing the production of lactic acid in the supplemented medium with the preliminary study, an increase of between 30 and 40 % can be observed in the supplement media, and significant differences (p-value < 0.05) were found in the statistical analysis.

On the contrary, the maximum acetic acid concentration (C_{max}) was reached later in the supplemented hydrolysates than in the no supplemented ones, being 1.92±0.07 g L^{-1} at 120 h in the medium supplemented with yeast extract, and 1.64±0.06 g L^{-1} at 168 h in the medium supplemented with peptone. These values are higher than in the preliminary study, obtaining significant differences (p-value < 0.05) among the three media tested.

The same conditions were tested with *L. casei*. The evolution of the LA concentration during fermentations is shown in Figure 3B. L_{max} was reached at 72 h of fermentation on both media, which is much earlier than in the *L. plantarum* fermentations. However, the value obtained was lower, being 6.20±0.62 g L^{-1} and 4.89±0.23 g L^{-1} for the hydrolysate supplemented with yeast extract and peptone, respectively. When the three media were compared, significant differences were found only in the medium supplemented with yeast extract (p-value > 0.05). The same effect is observed in the production of acetic acid. Thus, the C_{max} were 2.51±0.27 g L^{-1} in the hydrolysate supplemented with yeast extract and 1.93±0.19 g L^{-1} in the peptone supplemented medium. In both cases after 120 h of fermentation.

The pH measured during the fermentations of *L. plantarum* and *L. casei* is showed in Figure 3C and Figure 3D, respectively. These values follow a similar trend, but both were slightly lower than in the preliminary study with no supplement hydrolysates. The pH drops in the first 48 h to values lower than 4.5, that could explain the low consumption of reducing sugars which remains above 50 g L^{-1} at the end of fermentations in all cases.

All these results show that both strains require a nitrogen supplementation to improve the lactic acid yield on ESBPP hydrolysate. Moreover, lactic acid production was influenced by the nitrogen source employed in the *L. casei* fermentations, obtaining a higher LA concentration with yeast extract. De la Torre *et al*. studied the effect of the nitrogen source (yeast extract, peptone and meat extract) in the fermentation with *Lactobacillus delbrueckii* ssp. *delbrueckii* CECT 286 on MRS medium. In this study was concluded that peptone was not essential for lactic acid production with this strain[30]. However, this effect was not observed in the *L. plantarum* fermentations. Yeast extract provides nitrogen, amino acids and vitamins that are essential for the LAB growth[31]. Nevertheless, peptone is normally extracted from plant and animal tissues producing peptides with lacks minerals and/or vitamins compared with other nitrogen sources. For this reason, yeast extract is generally preferred than other nitrogen sources in LAB fermentations. The contrasting findings obtained with both strains may be due to differences in their metabolisms, being different the required nutrients in each case.

L. plantarum strain was selected for the following experiments because a higher L_{max} was obtained with this bacterium. Also, since no difference was found in the L_{max} for this microorganism when the hydrolysate was supplemented with yeast extract or peptone, yeast extract was used in the following studies, due to its lower price. In this way, it must be taken into account that the price of the produced lactic acid can be 1.7 times higher when peptone is added instead of yeast extract, according to the formula for the estimating LA price proposed by de la Torre *et al.*[30].

3.2.2 Effect of pH control

The production of lactic acid by LAB is influenced by the pH value in the culture medium. According to literature, the optimum pH for the lactic acid fermentation is between 5 and 7[11]. In previous experiments, as pH was not controlled, it decreases during the process to values not suitable for LAB growth. For this reason, two strategies for pH control were tested. In the first one, pH was controlled manually by adding NaOH 10 N (three times per day) to maintain the pH value at 6.5. In the second one, pH was controlled by adding calcium carbonate to the hydrolysate before fermentation, being tested the initial concentrations of 9, 18, 27, 55 and 75 g L^{-1}.

The evolution of glucose, arabinose, lactic acid, acetic acid, biomass concentration, and pH, through the fermentation time, is shown in Figure 4 for all the conditions tested. Firstly, it can be observed that glucose is consumed before than arabinose in all cases. This behaviour would be expected, because, according to literature, LAB metabolize first glucose rather than other sugars[6]. Thus, glucose is fully consumed in 48 h in the experiment with 9 g L^{-1} of $CaCO_3$ or in the experiment with NaOH regulation. For $CaCO_3$ concentrations higher than 9 g L^{-1}, glucose is depleted after 24 h of fermentation, indicating even faster fermentations. Regarding to arabinose, it is consumed after 72 h of fermentation in the experiments with NaOH regulation or with $CaCO_3$ concentrations of 27, 55 and 75 g L^{-1}; and not being depleted in the cases of 9 and 18 g L^{-1}. These results agree with the pH variation in the media, as pH values lower than 5 was reached in 24 h for the experiments with 9 and 18 g L^{-1} of $CaCO_3$.

The biomass increased as the sugars were consumed (Figure 4), reaching a maximum after 72 h of fermentation. After this time, a decrease can be observed in the experiments regulated with NaOH and $CaCO_3$ concentrations of 9, 18 and 27 g L^{-1}. However, the number of viable cells remained constant in the fermentations with 55 and 75 g L^{-1} of $CaCO_3$.

The maximum LA concentration (30 g L^{-1}) was reached with 9, 18 and 27 g L^{-1} of $CaCO_3$ and with NaOH, with no significant differences between them. However, it is obtained earlier in the case of 27 g L^{-1} (48h). On the other hand, the maximum LA concentration decreased a 16 % when 55 and 75 g L^{-1} of $CaCO_3$ was added in the fermentation medium. Kotzamanidis et. al, also observed a decrease in the lactic acid production when $CaCO_3$ concentrations higher than 70 g L^{-1} was added in the lactic acid fermentations of beet molasses by *Lactobacillus delbrueckii*[32]. Acetic acid is also produced as co-product due to the presence of arabinose in the hydrolysate. However, its production depends on the concentration of neutralizing agent added and the length of fermentation. The highest concentration of acetic acid (22 g L^{-1}) was obtained in the fermentation with NaOH. However, in the fermentations with $CaCO_3$, the concentration of acetic acid increased as the concentration of $CaCO_3$ does (Figure 4). In fermentations with NaOH and $CaCO_3$ concentrations higher than 27 g L^{-1} can be observed a decrease in lactic acid concentration after its peak was reached. In these fermentations, pH remains stable and, after sugars depletion, *L. plantarum* can metabolise part of lactic acid into acetic acid due to specific enzymes present in its metabolism[33–35]. Similar results were obtained by Quatravaux *et. al*, who performed a study with different aeration levels at a constant pH[36].

In addition of this, the yields obtained are showed in Figure 5. In this figure is represented the proportion of lactic acid yield and acetic acid yield that are obtained when the maximum lactic acid is produced and when the maximum acetic acid is produced. It can be observed that the total yield is higher in the moment when the maximum concentration of acetic acid is reached. However, higher proportion of acetic acid is obtained. This fact is interesting because the process can be stopped depending on the purpose of the process. If a pure stream of lactic acid is required, the process can be stopped earlier, but if a higher yield is required, the process can be stopped later. In this sense, the maximum proportion of lactic acid yield is obtained with 9 g L^{-1} of $CaCO_3$, while the maximum total yield is obtained with 27 g L^{-1} or NaOH, in both cases in the moment of maximum acetic acid production. In these last fermentations, a greater use of ESBPP is achieved although a mixture of both acids is obtained. For instance, both acetic and lactic acid can be used for polyhydroxyalkanoates production by fermentation with pure or mixture culture[37] or as a preservative in the food industry[38].

In summary, the process presented in this paper (see Figure 1) revalorizes a by-product of the sugar industry (ESBPP) to produce value-added products, such as, lactic and acetic acids. The use of beet pulp, instead of a synthetic culture medium with glucose as a carbon source, significantly reduces costs associated with the supply of the raw material because it is a cheap and renewable source[39]. In addition, in the present paper it is proposed a different use of sugar beet pulp than the usual one, which is animal feed. On the other hand, in the production of value-added products from lignocellulosic biomass, the cost of the enzymes used for the hydrolysis is the bottleneck of the process. To reduce the process cost, the enzymes are produced by SSF on ESBPP and this

fermented solid is added directly to fresh ESBPP for its hydrolysis. In this way, enzyme extraction and purification stages during the enzyme production process are avoided[19]. Therefore, a simpler and more environmentally friendly procedure to obtain the sugar hydrolysate is also proposed.

3.3 Kinetic model parameters

The kinetic model proposed in the Material and Methods section is based on the observed findings of the lactic fermentation of *Lactobacillus plantarum* on ESBPP hydrolysate with pH regulation. The three metabolic reactions observed, detailed in section 2.5, are the homofermentative conversion of glucose into lactic acid (eq. 1), the heterofermentative conversion of arabinose into lactic and acetic acids (eq. 2), and the oxidation of lactic acid into acetic acid (eq. 3). Reaction 1 and 2 were included considering that *L. plantarum* is classified in the literature as facultative heterofermentative strain[26,34,40–43]. Reaction 3 was included based on the statements of Kandler et al, Pintado et al. and Goffin et al.[33–35]. These authors observed that in the presence of oxygen and at low concentrations of sugars, *L. plantarum* convert lactic acid into acetic acid. The same results were observed in our study; thus, it has been considered in this work those three metabolic reactions into the model. The model uses simple equations to predict the evolution of substrates (glucose and arabinose), products (lactic and acetic acid), biomass and pH, during the fermentation on complex media as ESBPP hydrolysate, and the R^2 value obtained from fitting the model to the experimental data is in most of the cases in the rank of 0.95–0.99.

After the numerical fitting of the model parameters to the set of experimental data gathered, following the procedure that it has been described above, the values summarized in Table 1 were obtained. As it can be seen in previous figures, the growth of *Lactobacillus* strains is influenced by the medium pH, but the values of the disinfection constant obtained are very similar in all the ESBPP cases (0.1–0.2 $h^{-1}M^{-1}$), and besides in all the MRS cases (1.5–2.5 $h^{-1}M^{-1}$). Thus, the intrinsic pH effect is homogeneous, but the evolution of the acid concentration and the pH values are different in each case. Consequently, the addition of calcium carbonate as a neutralizing agent produces a variable pH during fermentations. Moreover, it causes a different behaviour of the microbial growth and acid production, obtaining different yields in each case.

Regarding the maximum specific growth rate of *Lactobacillus plantarum* based on glucose (μ_{Go}), it must be annotated that it presents a very narrow range (1.5–3.5 h^{-1}), even for the different types of medium tested (ESBPP hydrolysate and synthetic MRS). The specific rate based on arabinose (μ_{Ao}) also shows a narrow range (0.01–0.09 h^{-1}) but values are one hundred times lower than the previous one. So, growth on glucose is favoured over growth on arabinose. Only when arabinose is the unique carbon source in the medium, specific rates for glucose and arabinose are of the same order of magnitude (5.6 h^{-1}), showing that the cell metabolism is changed in those cases. Finally, the growth rate on lactic acid (μ_{Lo}) also presents a narrow range of values in practically all media. In the case of the ESBPP hydrolysate, this is ten times lower than the one for glucose (0.1–0.3 h^{-1}). The same occurs in the case of the synthetic MRS medium with arabinose (0.4 h^{-1}). However, in the case of the synthetic MRS medium with glucose, the specific rate is ten times greater (2–3 h^{-1}). Thereby, it seems that the arabinose presence reduces lactic

consumption. This fact could be useful to improve lactic acid production and gives a chance to arabinose wastes in front of other fermentation media in the lactic acid industry.

Monod constants for the three substrates (K_{MG}, K_{MA}, K_{ML}) present a clear dependency on the medium's pH. Thus, in the case of calcium carbonate experiments, these three parameters have a value close to 1 M at very low carbonate concentrations. They decrease when the carbonate concentration increases, and they fall into a value around 0.4 M for a carbonate calcium concentration of 27 g L^{-1}. For higher carbonate calcium concentrations take the same value, of around 0.4 M. Taking into account that a higher Monod constant means a lower affinity substrate-strain, it can be concluded that the use of calcium carbonate with ESBPP hydrolysates favours the consumption of the substrate (with the three carbon sources).

Concerning the substrate consumption coefficients (Y_{GX}, Y_{AX} and Y_{LX}), they take also very narrow ranges, showing no pH influence. As can be seen in Table 1, in the ESBPP media, the glucose coefficient has values lower values than the corresponding for arabinose, demonstrating that this bacterium needs more amount of arabinose than glucose to grow. Again, it is supported the idea that glucose metabolism is favoured versus arabinose.

The Y_{LG} coefficient, which represents the fraction of glucose converted to lactic acid by reaction 1. takes a narrow range of values between 0.75-0.90, without significant pH influence (see Table 1). Thereby, the assimilation of glucose to other aims is the

remaining amount ($1-Y_{LG}$), which keeps this in the range of 0.25–0.10. This means that a total conversion of glucose to acids is not possible in these conditions.

4 Conclusion

ESBPP hydrolysates can be used to produce lactic acid with high yield by lactic fermentation. Between both *Lactobacillus* species studied, *L. plantarum* produced a higher concentration of lactic acid than *L. casei*. Besides, the production of lactic acid can be improved by supplementing ESBPP hydrolysate with 5 g L^{-1} of yeast extract and by controlling the pH during fermentation. Acetic acid was also produced as co-product due to the presence of arabinose in the hydrolysate. Depending on the method used to regulate the pH, the proportion of lactic and acetic acid produced can be different. This fact can be useful depending on the subsequent process performed in this stream. Finally, the kinetic model developed shows good fitting parameters and allows to predict the evolution of substrates, products, biomass and pH.

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Figure legends

Figure 1. Flowchart of the process employed to produce lactic acid from exhausted sugar beet pulp pellets by enzymatic hydrolysis and lactic acid fermentation.

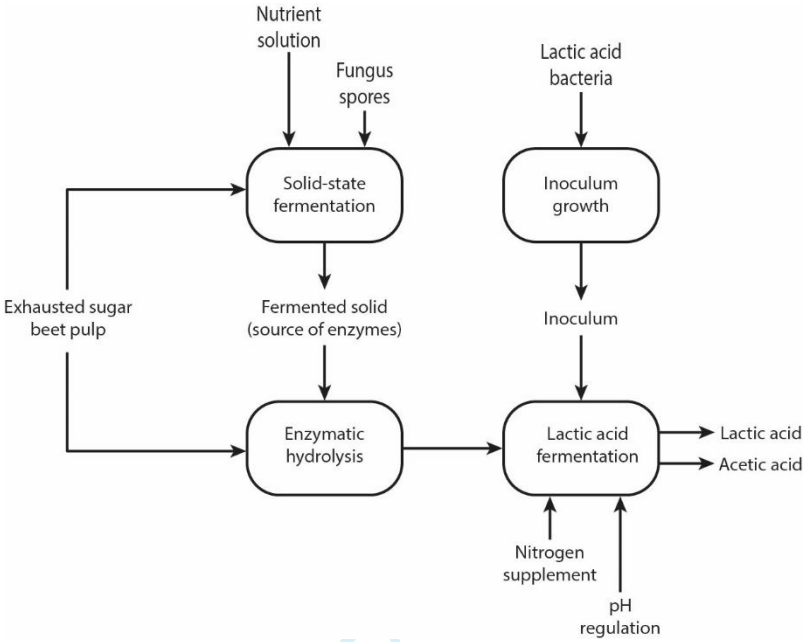
Figure 2. Evolution of simple sugars concentration in *L. plantarum* fermentation in MRS medium with glucose and pH regulation with NaOH (square), glucose and pH regulated with $CaCO_3$ (circle), arabinose and pH regulated with $CaCO_3$ (triangle), and, xylose and pH regulated with $CaCO_3$ (diamond).

Figure 3. Evolution of lactic acid concentration (g L^{-1}) and pH in *L. plantarum* (A, C) and *L. casei* (B, D) fermentations, respectively. So, without nitrogen supplementation (square), with 5 g L^{-1} of yeast extract (circle) and with 5 g L^{-1} of peptone (triangle).

Figure 4. Evolution of glucose (square), arabinose (circle), lactic acid (up-triangle) and acetic acid (down-triangle) concentrations, and pH (diamond) in *L. plantarum* fermentations, with different pH regulation methods: NaOH (A), 9 g L^{-1} $CaCO_3$ (B), 18 g L^{-1} $CaCO_3$ (C), 27 g L^{-1} $CaCO_3$ (D), 55 g L^{-1} $CaCO_3$ (E), 75 g L^{-1} $CaCO_3$ (F).

Figure 5. Maximum lactic acid yield ($Y_{Lmax/S}$), acetic acid yield ($Y_{C/S}$), maximum acetic acid yield ($Y_{Cmax/S}$) and lactic acid yield ($Y_{L/S}$) in the fermentations with various pH regulation methods.

Table 1. Kinetic parameters estimated for the lactic acid fermentation in three different mediums by *L. plantarum*: a) ESBPP hydrolysate; b) synthetic medium of MRS with glucose as main carbon source (MRS-glu); and synthetic medium of MRS with arabinose as main carbon source (MRS-ara). The pH was regulated by adding NaOH or a specific concentration of calcium carbonate.



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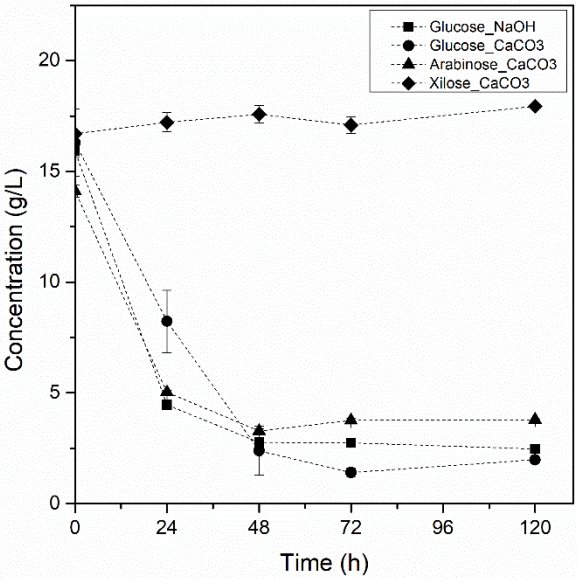

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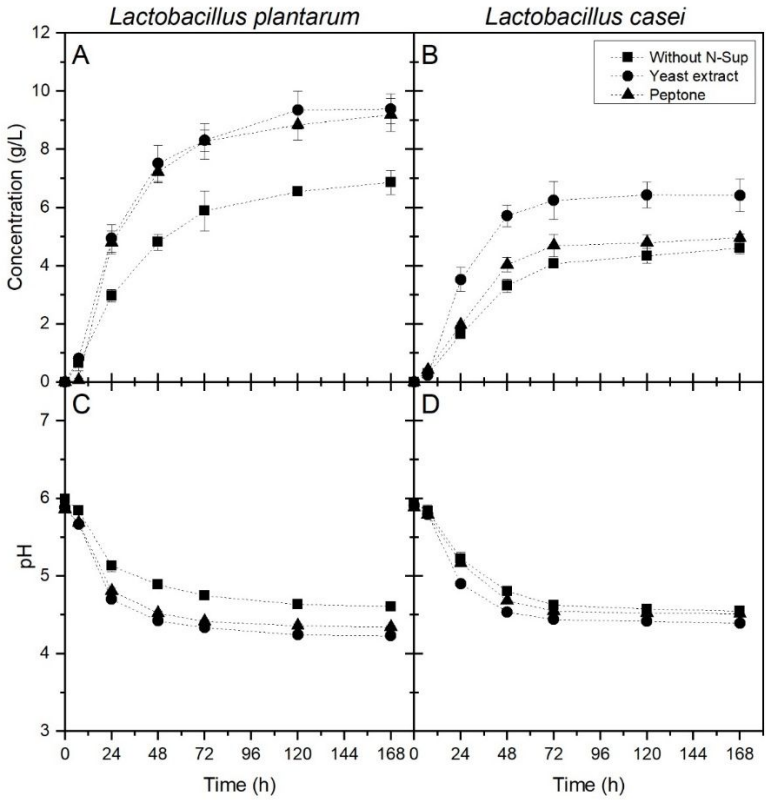

Figure 3. Evolution of lactic acid concentration (g/L) and pH in *L. plantarum* (A, C) and *L. casei* (B, D) fermentations, respectively. So, without nitrogen supplementation (square), with 5g/L of yeast extract (circle) and with 5g/L of peptone (triangle).

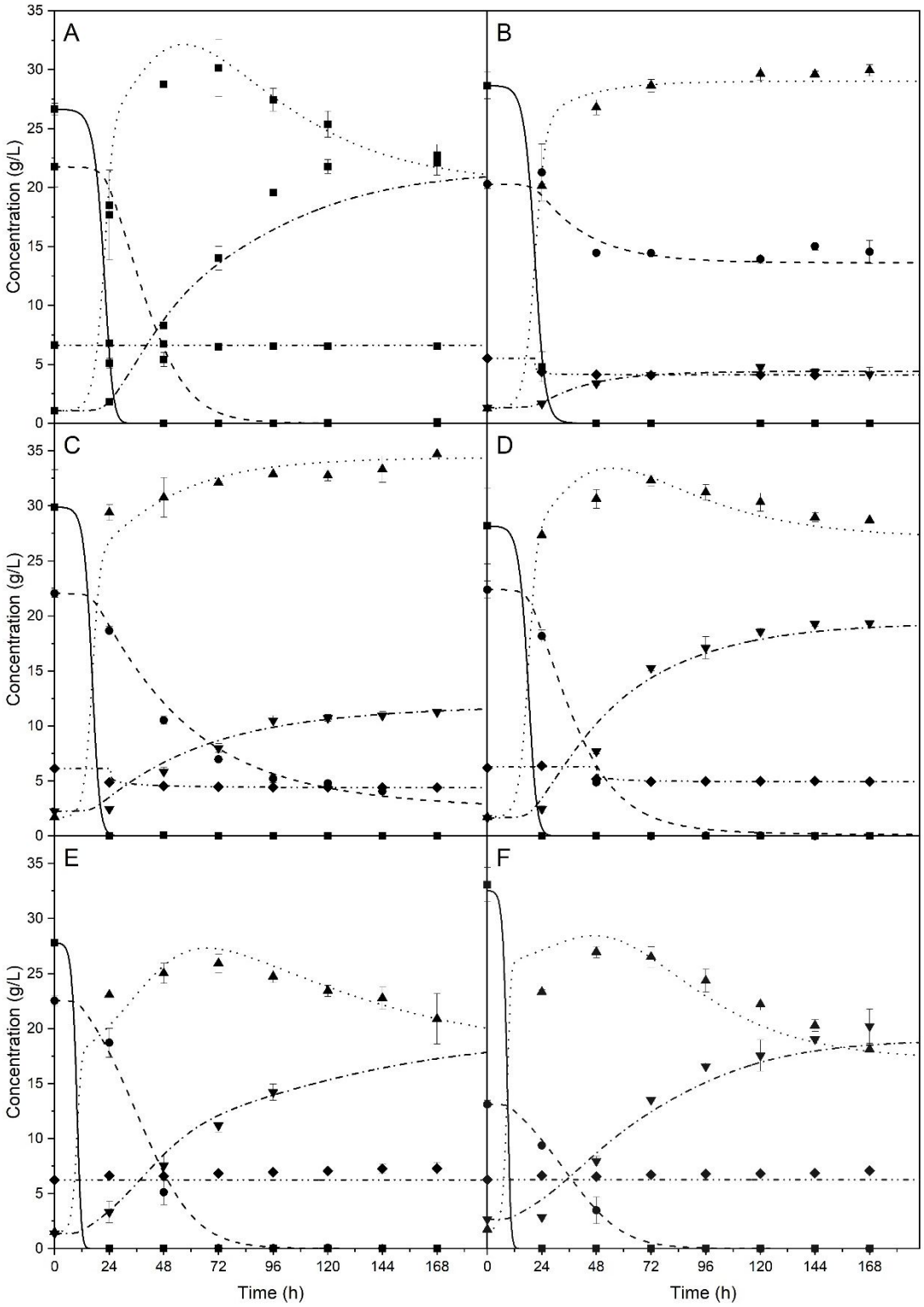

Figure 4. Evolution of glucose (square), arabinose (circle), lactic acid (up-triangle) and acetic acid (down-triangle) concentrations, and pH (diamond) in *L. plantarum* fermentations, with

different pH regulation methods: NaOH (A), 9 g/L $CaCO_3$ (B), 18 g/L $CaCO_3$ (C), 27 g/L $CaCO_3$ (D), 55 g/L $CaCO_3$ (E), 75 g/L $CaCO_3$ (F).

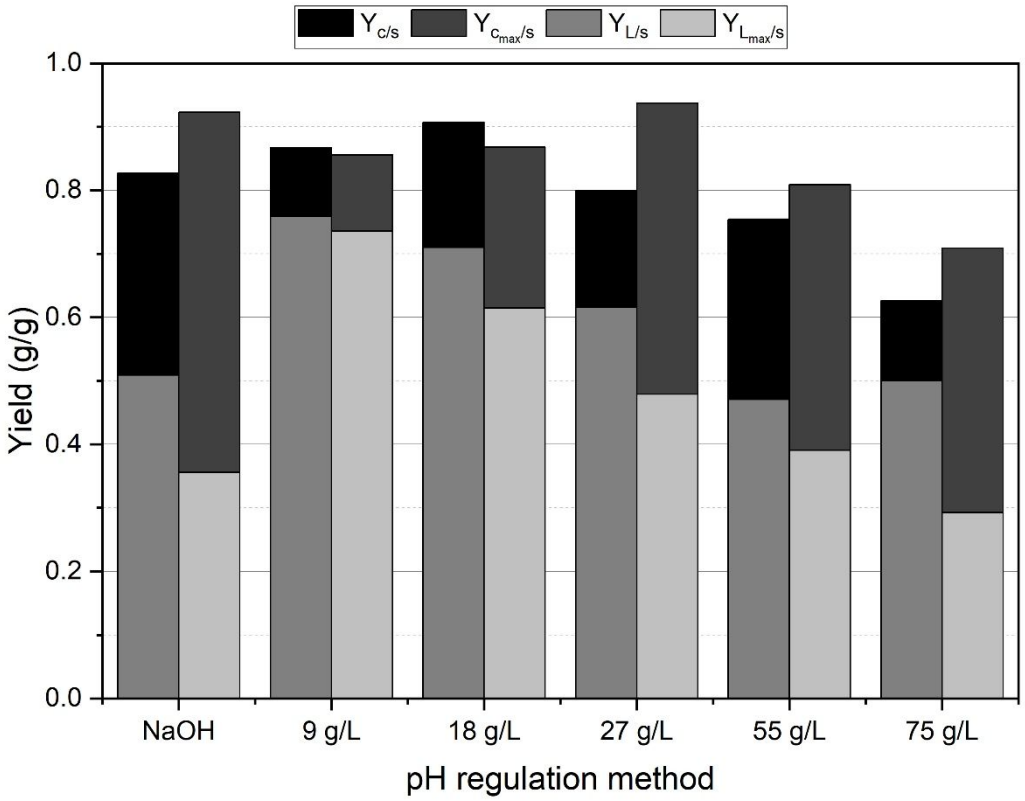

Figure 5. Maximum lactic acid yield ($Y_{Lmax/S}$), acetic acid yield ($Y_{C/S}$), maximum acetic acid yield ($Y_{Cmax/S}$) and lactic acid yield ($Y_{L/S}$) in the fermentations with various pH regulation methods.

Parameters (Units)		pH regulation method								
		ESBPP hydrolysate						MRS – gluc		MRS – ara
		9 g L^{-1} †	18 g L^{-1} †	27 g L^{-1} †	55 g L^{-1} †	75 g L^{-1} †	NaOH	NaOH	18 g L^{-1} †	18 g L^{-1} †
k_d	(1 $h^{-1}M^{-1}$)	0.192	0.081	0.188	0.162	0.262	0.206	2.459	1.739	1.658
μ_{Go}	(1 h^{-1})	2.973	3.412	3.113	3.062	3.612	1.543	2.911	3.344	–
μ_{Ao}	(1 h^{-1})	0.033	0.059	0.095	0.065	0.003	0.046	–	–	5.618
μ_{Lo}	(1 h^{-1})	0.129	0.099	0.335	0.188	0.326	0.250	3.982	1.687	0.413
Y_{LG}	(mol mol^{-1})	0.851	0.790	0.894	0.582	0.744	0.890	0.922	0.779	–
K_{MG}	(g L^{-1})	178.8	165.3	168.5	78.7	92.1	81.9	67.5	67.8	–
K_{MA}	(g L^{-1})	136.8	198.4	197.2	50.1	22.7	147.0	–	–	124.2
K_{ML}	(g L^{-1})	105.6	76.8	73.8	33.4	38.5	34.5	79.3	84.3	12.4
Y_{GX}	(µmol $Gcell^{-1}$)	0.505	0.506	0.503	0.799	0.799	0.623	0.162	0.009	–
Y_{AX}	(µmol $Gcell^{-1}$)	1.375	1.374	1.309	0.430	4.301	2.587	–	–	0.099
Y_{LX}	(µmol $Gcell^{-1}$)	0.030	0.028	0.027	0.011	0.014	0.022	0.464	0.083	0.219
R^2	(–)	0.959	0.978	0.988	0.986	0.978	0.980	0.907	0.879	0.852

† Concentration of $CaCO_3$

Table 1. Kinetic parameters estimated for the lactic acid fermentation in three different mediums by *L. plantarum*: a) ESBPP hydrolysate; b) synthetic medium of MRS with glucose as main carbon source (MRS–glu); and synthetic medium of MRS with arabinose as main

carbon source (MRS-ara). The pH was regulated by adding NaOH or a specific concentration of calcium carbonate.