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ORIGINAL ARTICLE

Production of D-lactic acid by *L. delbrueckii* growing on orange peel waste hydrolysates and model monosaccharide solutions: effects of pH and temperature on process kinetics

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Abstract
D-lactic acid is a key monomer of polylactic acid (PLA) and other biopolymers. In this work, the effects of temperature and pH on the D-lactate production with *Lactobacillus delbrueckii* in model sugar solutions and real orange peel waste hydrolysates (OPWH) have been studied and modeled with a non-structured non-segregated kinetic model. *Lactobacillus delbrueckii* ssp. *delbrueckki* CECT 286 yields 0.88 g/g of D-lactate (purity 97%) with a productivity of 2.56 g/L·h in the best tested conditions (40 °C and pH 5.8). The proposed kinetic model is able to describe the performed runs, and its kinetic parameters reflect the influence of temperature and pH on the uptake of monosaccharides, D-lactic acid production, and bacterium growth. Moreover, it can be applied to simulate runs with real OPWH supplemented with MRS broth or corn steep liquor (CSL) as a nitrogen source.

Keywords D-lactic acid · Orange peel waste hydrolysates · pH · Temperature · Kinetic model · *Lactobacillus delbrueckii*

1 Introduction

Lactic acid is a hydroxycarboxylic acid that presents two enantiomeric forms: D and L. It has a wide range of applications in food, pharmaceutical, and cosmetic industries [1]. In the last decades, lactic acid is being applied also as monomer for the production of biodegradable and biocompatible plastics, namely poly-lactic acid polymers (PLA) [2–5], with good mechanical, thermal, and gas and water barrier properties. Therefore, their market is increasing at a yearly growth rate of near 19% and a prospective annual production volume of higher than 1.2 million tons by 2020. The physical properties of these polymers are highly dependent on the percentage and distribution of the enantiomers [1, 6]: PLA constituted by a racemic mixture of isomers is less stable than PLA produced from pure D- or L-lactic acid (PDLA and PLLA), while PDLA has also a higher melting temperature than PLLA. This fact explains why research works have been focused on D-lactic acid production [7, 8].

Lactic acid can be synthesized by chemical or fermentation processes. In the first case, the process starts with the hydrolysis of lactonitrile, a compound obtained from oil, yielding a racemic mixture of lactic acid [9]. The biotechnological production, in comparison, has some advantages, as it can provide pure lactic acid isomers at lower costs, avoiding their purification from the racemic mixture. Although the bioproduction of L-lactic acid is realized nowadays at large industrial scale by several companies (NatureWorks LCC, Corbion Purac, Galactic, Henan Jindan Lactic Acid Co. Ltd., and more), the number of studies focused on the bioprocess to obtain the D-enantiomer is relatively scarce [10–12]. At industrial scale, NatureWorks has recently licensed the Optipure® process developed by Plaxica, a process including distillation after an enzymatic resolution of the isomers to obtain pure D-lactic acid, while Corbion Purac has licensed a fermentation technology from Myriant and started to produce D-lactate since 2008 [13]. The main problem of this bioprocess is its low profitability, mostly derived from the raw material cost [3, 4, 14]. Therefore, inexpensive raw materials are needed to increase the economic feasibility of this fermentative process [13, 14].

Most studies on literature focusing on lactic acid production aim to search for novel substrates from renewable resources. Several materials have been considered as optional

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feedstock, such as byproducts of agriculture industries, food industries or waste such as lignocellulosic biomass, whey, and raw glycerol produced in excess in the biodiesel process [2, 4, 5, 9, 15]. Renewable raw materials are rich in cellulose and, as a consequence, in glucose as the monomer. However, cellulose macrofibers are linked by hemicellulose, lignin and/or pectin, while non-macromolecular compounds are also components of such feedstock. Therefore, a complex mixture of monosaccharides and other compounds is obtained when they are properly pretreated and hydrolyzed. This is the main reason behind the studies in literature focused on the effect of mixed sugars [16–19]. Taking into account the amount of wastes created during fruit consumption and food processing, orange peel waste (OPW) and pulp waste are generated every year in huge amounts [20]. This waste presents a high amount of free and bound monosaccharides, namely glucose, fructose, galactose, and arabinose [21].

Lactic acid bacteria (LAB), which includes genera like *Enterococcus, Lactococcus*, *Sporolactobacillus,* and *Lactobacillus* can produce D-lactic acid from renewable resources. Batch, fed-batch, repeated batch, and continuous processes are employed to improve the fermentative lactic acid production [4]. To operate these fermentation processes, the main variables that affect cell growth, lactic acid concentration, yield, and productivity have to be optimized, with temperature and pH being the most important among these variables [2]. According to Prasad et al. [22], *L. lactis* is able to produce 24.3 g/L of D-lactic acid with a purity > 98% from casein whey permeate under controlled pH conditions. *L. delbrueckii* produces high purity D-lactic acid from sugarcane juice with a yield and productivity of 0.95 g/g and 1.66 g/(L·h), respectively [7]. Changes in pH can deactivate the pyruvate pathway (pyruvate to lactate or to byproducts like acetoin acetate or ethanol); and pH seems to affect the shift towards the D- or L-isomer, due to L(+)-lactate dehydrogenase and D(−)–lactate dehydrogenase activities [23].

In industrial bioprocesses, mathematical models are employed to predict, to monitor, and to improve both the properties and behavior of these processes [24]. A good understanding of the bioprocess leads to adequate kinetic models that allows to predict the behavior to external perturbations [24]. Some authors have proposed several kinetics models that include substrate and product inhibition to describe the dynamics of lactic acid processes [25–27]. Kwan et al. [27] use *S. thermophiles* and *L. casei* to study glucose and fructose inhibition as well as the inhibition due to the product (lactic acid). Subsequently, they proposed a kinetic model able to predict the production of this acid from different food waste in a process that achieved a yield of up to 0.94 g/g and a productivity of 2.61 g/(L·h). Most kinetic models proposed in literature for lactic acid production consider the inhibitory effect of the product to describe the growth and the consumption of substrate: if lactic acid increases in the broth, the internal—or cytoplasmic—and external pH levels drop dramatically, if no buffering or pH control is present, reducing cell viability and deactivating several metabolic pathways. Also, H^+ concentration is used to describe the process and the pH value effect on biomass [28]. On the other side, the complexity of the mixture of carbohydrate substrates should be considered when proposing a kinetic model on the use of renewable sources [27]. Schmitt et al. proposed a kinetic model to fit the acetic acid production and growth of *Moorella thermoacetica* using a mixture of glucose and xylose, which is able to explain the differences in the process when more than one sugar is present [29]. Also, Lameiras et al. studied the kinetics and the effect of stoichiometry on *Aspergillus niger* growth on six different sugars (glucose, xylose, mannose, rhamnose, arabinose, and galacturonic acid). They proved the need to consider the role of the different substrates in the design of a fermentation process based on plant wastes feedstock [30]. Nevertheless, sometimes, these models require a large number of parameters [31], a lumping of the substrates could be a good strategy in order to simplify the description of their time courses, as Choi et al. described in their work [32].

Due to the important effects of both process variables and mixtures of sugars on any bioprocess based on the use of renewable resources, this work aims on analyzing the influence of pH and temperature—two key process variables—on the uptake of glucose, fructose, galactose, and arabinose (a sugar mixture in orange peel waste hydrolysates—OPWH) on D-lactic acid production by *Lactobacillus delbrueckii* ssp. *delbrueckii* CECT 286. A kinetic model will be proposed in order to predict both the influence of the variables and the time course of the bioprocess and applied to mixtures resembling OPWH and also to real OPWH.

2 Materials and methods

2.1 Strain, inoculum preparation, and raw material

The microorganism used in this study is *Lactobacillus delbrueckii* ssp. *delbrueckii* CECT 286, provided by Biopolis S.L. (Paterna, Valencia, Spain). The medium employed for both the preinoculum and the inoculum cultures was MRS broth (glucose 20 g/L, peptone 10 g/L, meat extract 8 g/L, yeast extract 4 g/L, triammonium citrate 2 g/L, sodium acetate· $3H_2O$ 2 g/L, K_2HPO_4, 2 g/L, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$, and Tween 80). The pre-inoculum (4 h) and inoculum (12 h) cultures were carried out in 60-mL bottles filled with 50 mL MRS at 37 °C and 200 rpm in an orbital shaker, without pH control. The initial biomass concentration (inoculum) for all the performed runs was 0.1 g/L, carried out both in bottles and in bioreactor.

The orange peel wastes (OPW) employed for the experiments were provided by Biopolis S.L (Paterna, Valencia) and

they were hydrolyzed according to de la Torre (2017) [21]. Briefly, an adequate amount of OPW with 66% humidity is mixed with 20 mL of citrate buffer 50 mM pH 5.0 (up to 10% *w*/*w* DS—dry biomass), heated to 50 °C, and stirred at 300 rpm with a 6-flat blade turbine; subsequently, Celluclast 1.5 L (6 μL/g DS), Novozym 188 (3 μL/g DS), and Pectinex Ultra SP-L (7 μL/g DS) were added and the saccharification was let to proceed for 48 h. Finally, the remaining solid was removed by centrifugation at 9000 rpm for 10 min and the orange peel waste hydrolysate (OPWH) was sterilized by filtration and stored at − 4 °C until use.

2.2 Growth conditions and procedure in orbital shaker

To perform the temperature study (35, 37, 40, 43, and 47 °C), the experiments were carried out in triplicate using 100-mL bottles filled with 50 mL of MRS medium supplemented with 10 g/L of glucose, 20 g/L of fructose, 4 g/L of galactose, and 4 g/L of arabinose in order to simulate the sugar composition in OPWH [21]. The shaking rate was 200 rpm. Before inoculation, 50 g/L of $CaCO_3$ was added to the culture media for pH control. Afterwards, the medium was bubbled with nitrogen for several minutes after sealing the bottles with rubber caps and with the aid of two needles to ensure anaerobic conditions. Several samples were withdrawn from the bottles at different times in order to quantify the biomass, substrates, and product concentrations (a nitrogen balloon was used to keep anaerobic conditions).

2.3 Growth conditions and procedure in STBR

The experiments—in duplicates—o study the pH influence were performed in a STBR BIOSTAT B-Plus bioreactor (Sartorius AG, Germany) using a work volume of 2 L. The medium employed was the same as the former for the temperature study or a real OPWH; in both cases, medium was also bubbled with nitrogen to ensure anaerobic conditions before inoculation (nitrogen bubbling was turned off just before, so small volumes of air could enter the system afterwards, creating microaerobic conditions). In the bioreactor, pH was controlled at 5, 5.2, 5.5, 5.8, 6.0, and 6.2 by automatic addition of 5 M NaOH and/or 2 M HCl. Temperature was kept constant at 40 °C and the stirrer speed (a six flat bladed Rushton turbine) was set at 200 rpm. Several samples were withdrawn during each run to determine the time course of biomass, substrates, and product. The values were corrected taking into account the dilution due to the volume of base added for pH control.

2.4 Analytical methods

The concentrations of the different substrates and the lactic acid were measured by HPLC (Agilent Technologies 1100 Series, USA) equipped with a Refractive Index Detector at 55 °C. A Rezex ROA-Organic Acid H^+ (8%) column (300 × 7.8 mm; Phenomenex, USA) was used at 80 °C. The mobile phase was 0.005 M H_2SO_4 at a flow rate of 0.5 mL/min. D-lactic acid optical purity was also analyzed by HPLC using a DAD detector and a Chirex 3126 (D)-penicillamine (250 × 4.6; Phenomenex, USA) column working at room temperature. In this case, $CuSO_4$ 1 mM was used as mobile phase at 1.2 mL/min flow rate. Biomass concentration was quantified by UV-Vis spectrophotometry as optical density at 600 nm (Shimadzu UV-visible spectrophotometer UV-1603, Japan), traduced into biomass concentration by means of a calibration curve.

2.5 Mathematical methods

In order to determine the influence of the studied variables on the production, some parameters were calculated: titer ($C_{lac}{}^{max}$), acid yield ($Y_{P,S}$)—eq. (1)—and productivity (P) of D-lactic acid—eq. (2).

$$Y_{P,S} = \frac{C_{lac}^{max}}{C_{Consumed\ substrates}} \tag{1}$$

$$P = \frac{C_{lac}^{max}}{t} \tag{2}$$

The temporal evolution of the concentrations of all relevant components in all the runs leads to the proposal of a non-structured kinetic model considering two reactions. The first one (r_1) includes the consumption of glucose for producing both cells and D-lactic acid (eq. 3) and is limited by the glucose concentration, as expressed in eq. 5. The second one (r_2) is valid only when glucose is exhausted in the broth and it is related to the consumption of fructose + galactose (eq. 4), which are employed only for production. Its rate is influenced by the concentration of these monosaccharides (eq. 6). Therefore, the kinetic model can be represented by the following reaction scheme:

$$\nu_{G,X}\ G \xrightarrow{X} X + \nu_{P,X}\ P; r_1 \therefore G \neq 0 \tag{3}$$

$$\nu_{F+Gl,P}\ (Gl + F) \xrightarrow{X} P; r_2 \therefore G = 0 \tag{4}$$

Being their kinetic equations:

$$r_1 = \frac{\mu_m\ C_G\ C_X}{K_s + C_G} \tag{5}$$

$$r_2 = k_p\ C_{F+Gl} C_X \tag{6}$$

Then, the evolution of the considered components with time in the model has to be represented by two sets of differential equations depending on the presence or absence of glucose (glucose concentration minor than 0.1 g/L) in the broth:

Table 1 Influences of temperature (in stirred bottle at pH 5.4), pH (in bioreactor at 40 °C), and substrate (real OPWH with MRS and CSL broths, at 40 °C and pH 5.8) on the titer (C_P^{max}), yield ($Y_{P,S}$), productivity (P), and purity (% D-LA) of D-lactic acid, and on growth rate ($\mu_{biomass}$) and on glucose uptake rate (R_G) in the exponential phase (4–8 h)

RUN	T (°C)	CP^{max} (g/L)	YP,S (g/g)	P (g/L·h)	% D-LA	$\mu_{biomass}$ (gX/(L·h))	RG (gG/(L·h))
1	35	44.3 ± 1.2	0.84 ± 0.08	1.84 ± 0.12	95.5 ± 2.1	0.9 ± 0.1	4.5 ± 0.7
2	37	50.5 ± 1.7	0.85 ± 0.11	2.10 ± 0.18	95.9 ± 1.9	1.2 ± 0.2	7.5 ± 0.8
3	40	51.9 ± 1.4	0.88 ± 0.09	2.16 ± 0.17	95.9 ± 1.8	1.2 ± 0.2	7.5 ± 0.6
4	43	49.3 ± 1.5	0.84 ± 0.08	2.05 ± 0.09	93.9 ± 2.2	1.2 ± 0.1	6.2 ± 0.4
5	47	39.1 ± 1.6	0.82 ± 0.06	1.68 ± 0.08	93.4 ± 2.3	0.6 ± 0.1	2.5 ± 0.3
	pH	C_P^{max} (g/L)	$Y_{P,S}$ (g/g)	P (g/L·h)	% D-LA	$\mu_{biomass}$ (g_X/(L·h))	R_G (g_G/(L·h))
6	5.0	38.6 ± 1.4	0.75 ± 0.07	1.60 ± 0.17	98.4 ± 2.3	1.0 ± 0.2	5.5 ± 0.7
7	5.2	42.1 ± 1.3	0.75 ± 0.05	1.76 ± 0.14	97.5 ± 1.8	1.1 ± 0.2	7.1 ± 0.5
8	5.5	49.7 ± 1.8	0.87 ± 0.04	2.06 ± 0.09	98.5 ± 1.9	1.7 ± 0.3	8.5 ± 0.9
9	5.8	51.3 ± 1.6	0.88 ± 0.08	2.56 ± 0.32	97.3 ± 1.4	2.7 ± 0.3	12.5 ± 1.3
10	6.0	49.8 ± 1.5	0.87 ± 0.09	2.49 ± 0.08	97.1 ± 1.6	2.5 ± 0.2	10.5 ± 1.1
11	6.2	50.4 ± 1.3	0.87 ± 0.12	2.52 ± 0.11	98.1 ± 2.1	2.3 ± 0.2	9.5 ± 0.8
12	OPWH-MRS	48.6 ± 1.6	0.86 ± 0.06	2.02 ± 0.14	97.7 ± 1.8	2.7 ± 0.2	11.5 ± 0.8
13	OPWH-CSL	49.6 ± 1.8	0.83 ± 0.06	1.71 ± 0.07	98.1 ± 1.7	0.9 ± 0.1	5.8 ± 0.6

Note: OPWH-MRS stands for orange peel hydrolysate with MRS broth components

OPWH-CSL means orange peel hydrolysate supplemented with corn steep liquor

While there is glucose in the broth:

$$- \text{ Growth} : \frac{dC_X}{dt} = +r_1 \tag{7}$$

$$- \text{ Substrate consumption} : \frac{dC_G}{dt} = -\nu_{G,X} \cdot r_1 \therefore \frac{dC_{F+Gl}}{dt} = 0 \tag{8}$$

$$- \text{ D-lactic acid production} : \frac{dC_P}{dt} = +\nu_{P,X} \cdot r_1 \tag{9}$$

When glucose is exhausted in the broth:

$$- \text{ Growth} : \frac{dC_X}{dt} = 0 \tag{10}$$

$$- \text{ Substrates consumption} : \frac{dC_G}{dt} = 0 \therefore \frac{dC_{F+Gl}}{dt} = -\nu_{F+Gl,P} \cdot r_2 \tag{11}$$

$$- \text{ D-lactic acid production} : \frac{dC_P}{dt} = +r_2 \tag{12}$$

All data obtained for pH and temperature studies were used to build up the kinetic model. This model was fitted to data from each run employing numerical algorithms implemented in Aspen Custom Modeler® v10 sofware; namely, Levenberg-Marquardt non-linear regression (NL2SOL) coupled to 4th order Runge-Kutta numerical integration of the set of differential ordinary equations (DOE) [33].

To determine the goodness-of-fit of the proposed kinetic model, several parameters were considered: RMSE (the lower its value, the better the model fits to data), F-test, and the percentage of variation explained (%VE) (again, the highest percentage, the better the fit is). These values have been calculated by means of the following equations:

$$RMSE = \sqrt{\frac{SSR}{N-M}} \tag{13}$$

$$F = \frac{\sum_{i=1}^{N} \left(\frac{y_{i,calc}}{M}\right)^2}{\sum_{i=1}^{N} \frac{SSR}{N-M}} \tag{14}$$

Where SSR is the sum of squared residuals, N is the number of experimental data, M is the number of parameters of the proposed kinetic model, and $y_{i,calc}$ is the calculated value of the variable.

$$VE(\%) = 100\left(1 - \frac{\sum_{l=1}^{L} SSQ_l}{\sum_{l=1}^{L} SSQ_{mean_l}}\right) \tag{15}$$

Where SSQ_l is the sum of squared residues with respect to the calculated value of "l" variable and SSQ_{meanl} is the sum of deviation squares between experimental and the mean score with respect to the calculated values of the variable [34].

3 Results and discussion

3.1 Analysis of yields and productivities

As previously commented, to study the effect of temperature, five experiments at 35, 37, 40, 43, and 47 °C were carried out. The values of the titer, yield, and productivity of D-LA and of growth rate and glucose uptake rate for these runs are shown in Table 1. They all reach a maximum at 40 °C. Moreover, the

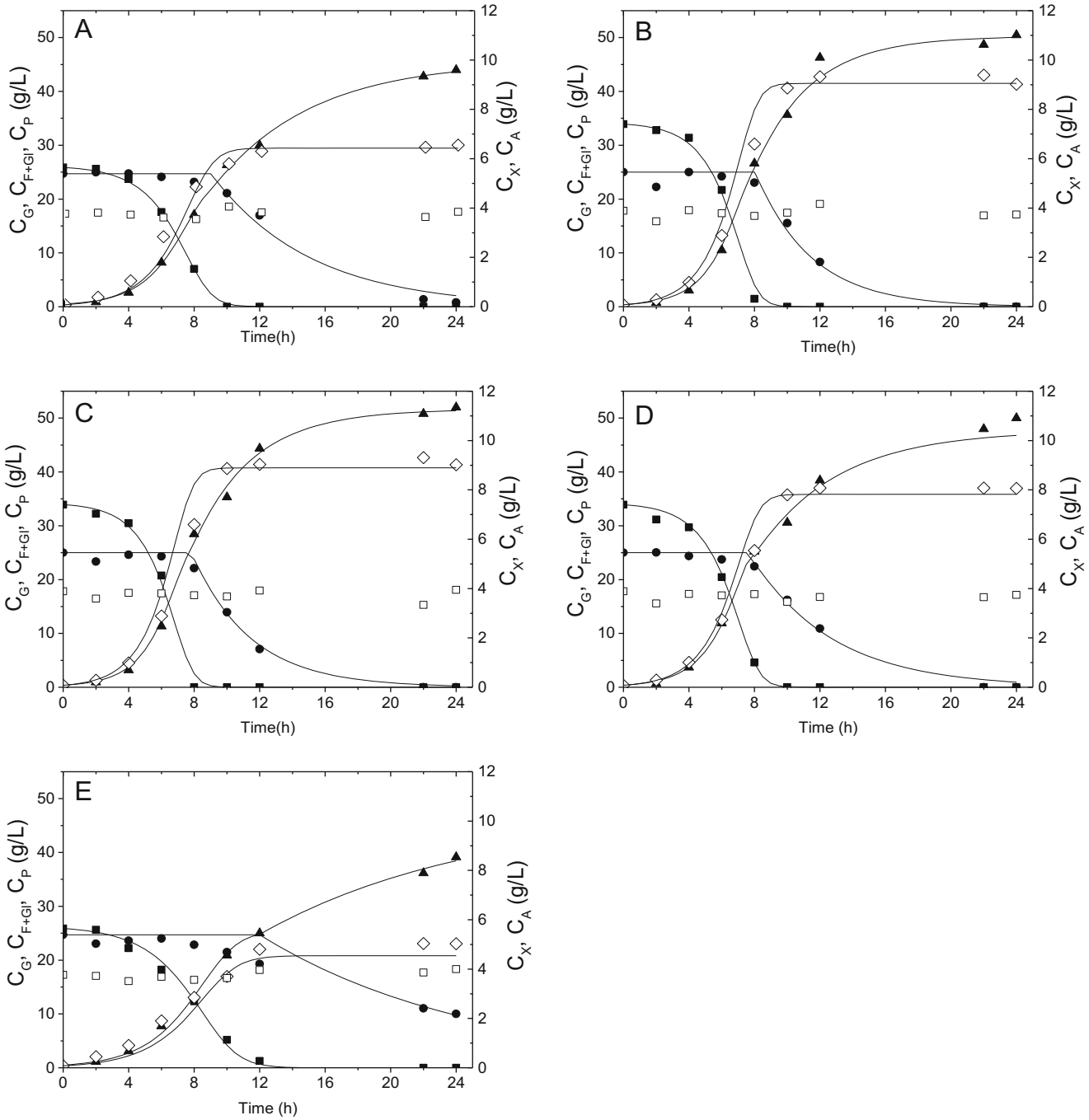

Fig. 1 Experimental data (points) and kinetic model predictions (lines) for the D-lactic acid production at different temperatures: 35 °C (**a**), 37 °C (**b**), 40 °C (**c**), 43 °C (**d**), and 47 °C (**e**). Data points: glucose (squares), fructose + galactose (circles), lactic acid (triangles), biomass (open diamonds), and arabinose (open squares)

percentage of D-LA is higher than 93% in all cases, reaching the best value at 40 °C.

Figure 1 indicates how the concentration evolves with time for D-lactic acid, glucose, fructose + galactose and biomass for the temperature study. Results show that arabinose is not metabolized by the microorganism, regardless of the temperature value, while glucose is completely consumed (up to 30 g/L) in all cases. In fact, this monosaccharide seems to be a growth-limiting nutrient. Also, it is evident that growth is clearly affected by temperature: the maximum concentration of biomass was obtained from 37 to 43 °C. Also, the higher the rate of glucose uptake is, the higher the growth rate is, as well as the concentration of biomass in the stationary phase. Regarding fructose and galactose uptake, these substrates are not consumed while glucose still remains in the broth but are completely consumed within 24 h in the temperature range

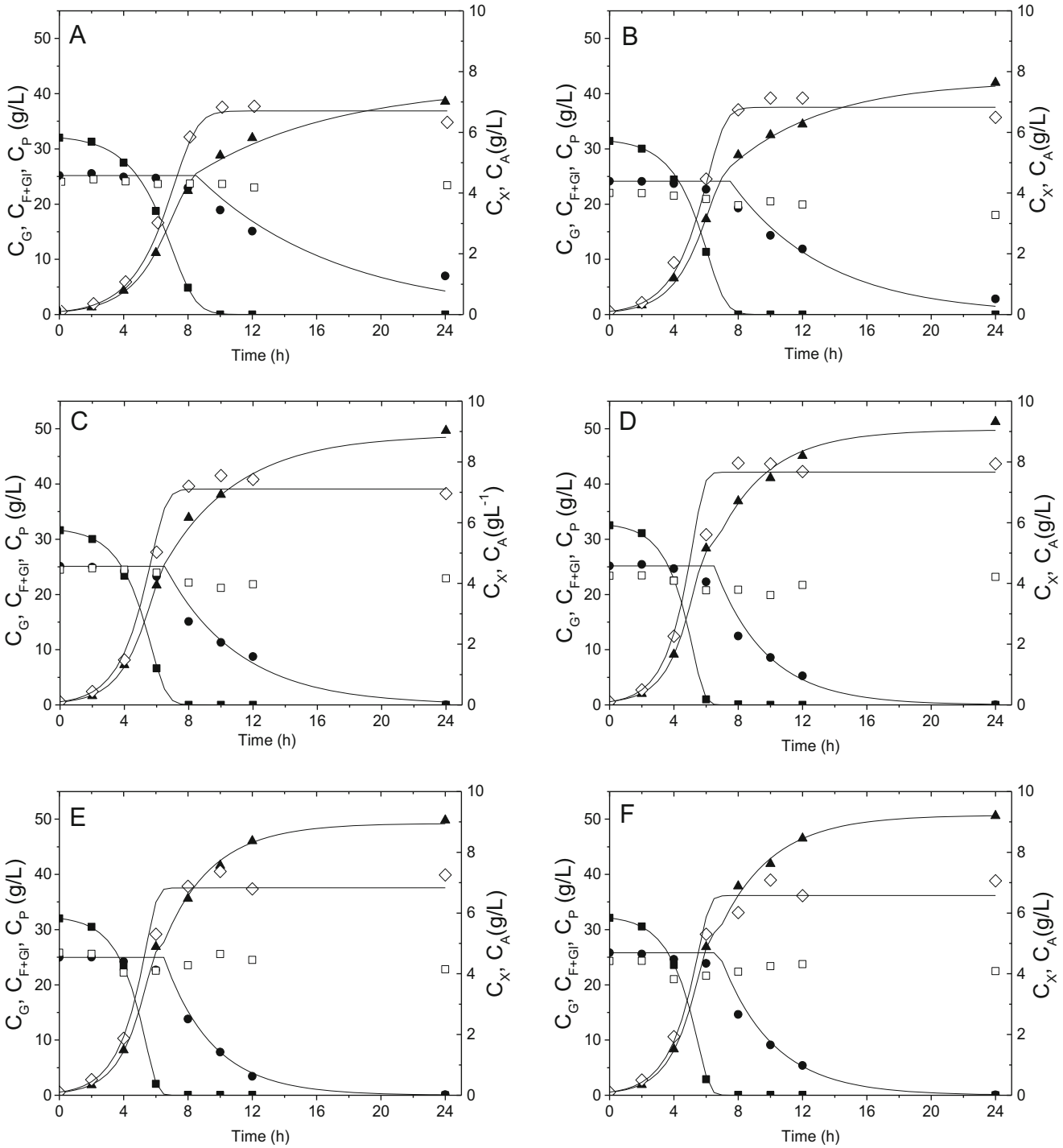

Fig. 2 Experimental data (points) and kinetic model predictions (lines) for the D-lactic acid production from different pH value: pH 5 (**a**), pH 5.2 (**b**), pH 5.5 (**c**), pH 5.8 (**d**), pH 6 (**e**), and pH 6.2 (**f**). Data points: glucose (squares), fructose + galactose (circles), lactic acid (triangles), biomass (open diamonds), and arabinose (open squares)

between 35 and 43 °C. When temperature rises over 43 °C, glucose uptake rate is much slower, while growth rate decreases dramatically due to heat shock. The lower viability of the cells is concomitant with lower productivity, titer, and yield of D-LA; exactly the opposite to the situation at 40 °C, where high rates related to biomass production and glucose uptake are reflected on a very active metabolism that produces 25% more D-LA compared to results at 47 °C. However, temperature affects much more substrate uptake and growth rates than D-LA production: the first parameter reduces to one third of its value at 40 °C when working at 47 °C, while the latter one reduces to a half.

Table 2 Statistical parameters obtained from all the fits to the proposed kinetic model performed in this study

RUN	Temperature (°C)	Statistical parameters			
		RMSE	VE (%)	F_{calc}	F_{tab}
1	35	0.63	99.67	5076	2.305
2	37	1.20	99.21	1195	2.305
3	40	0.93	99.53	3312	2.305
4	43	1.03	99.32	2410	2.305
5	47	1.28	98.03	1168	2.305
	pH	RMSE	VE (%)	F_{calc}	F_{tab}
6	5.0	1.01	99.04	1479	2.305
7	5.2	0.89	99.31	1894	2.305
8	5.5	0.95	99.31	2174	2.305
9	5.8	0.73	99.62	3182	2.305
10	6.0	0.57	99.76	5200	2.305
11	6.2	0.55	99.78	5236	2.305
13	Real OPWH with CSL	0.99	99.30	4025	2.210

The effect of pH was studied performing six runs, with their duplicates, in a 2-L work volume stirred tank bioreactor at six controlled pH values from 5 to 6.2. As in the case of temperature, D-lactic acid titer, yield, productivity, and purity, as well as growth rate and glucose uptake rate in the exponential phase, are determined and the results are collected in Table 1. The highest values of both D-lactic acid titer (51.3 g/L) and productivity (2.56 g/L·h) are achieved at a pH value of 5.8. At pH values equal or higher than 5.5, the yield does not seem to be affected by this variable. The purity in D-LA is higher than 97% for all the runs performed, and again, there is no influence of pH within the range tested. This higher purity could come from using a soluble base to control pH, avoiding racemization due to impurities in $CaCO_3$, the solid base employed in the temperature runs. Regarding growth rate and glucose uptake rate, they reach the best tested values at pH 5.8 and decreasing slightly at higher pH. However, pH lower than 5.8 rapidly decreases the values of both parameters, probably a consequences of problems due to internal pH control. In fact, rate parameters related to cell growth and glucose uptake are affected by pH much more deeply than those related to D-LA production, a trend similar to the one observed in the temperature runs.

Figure 2 reflects the evolution of the concentration profiles at different pH values: this variable seems to show a lower effect on growth compared to that shown by temperature. Again, arabinose is not consumed at all. In addition, the highest rate for glucose uptake (it is completely consumed between 6 and 9 h from the beginning of the fermentation) yields the highest growth rate and concentration of biomass in the stationary growth phase and seems to be the growth-limiting nutrient. Regarding the consumption of fructose and galactose, they are completely consumed in 24 h at pH values higher than 5.2. These monosaccharides are only used by the microorganism to produce D-LA, while glucose participates both in growth and D-LA production.

In both sets of experiments, there is a clear distinction between the consumption of glucose, the fastest one, and that one of fructose + galactose; this fact is probably due to a different rate and/or a competition of the membrane transporters of both sugars [35]. While glucose readily enters glycolysis, galactose needs prior isomerization to glucose after its phosphorylation, according to the Leloir pathway, or is modified via the tagatose pathway [36]. Galactose can be excreted in the presence of glucose, to be transported later again and subsequently transformed by the cell metabolism. In the case of fructose, its metabolism in homofermentative LAB is very

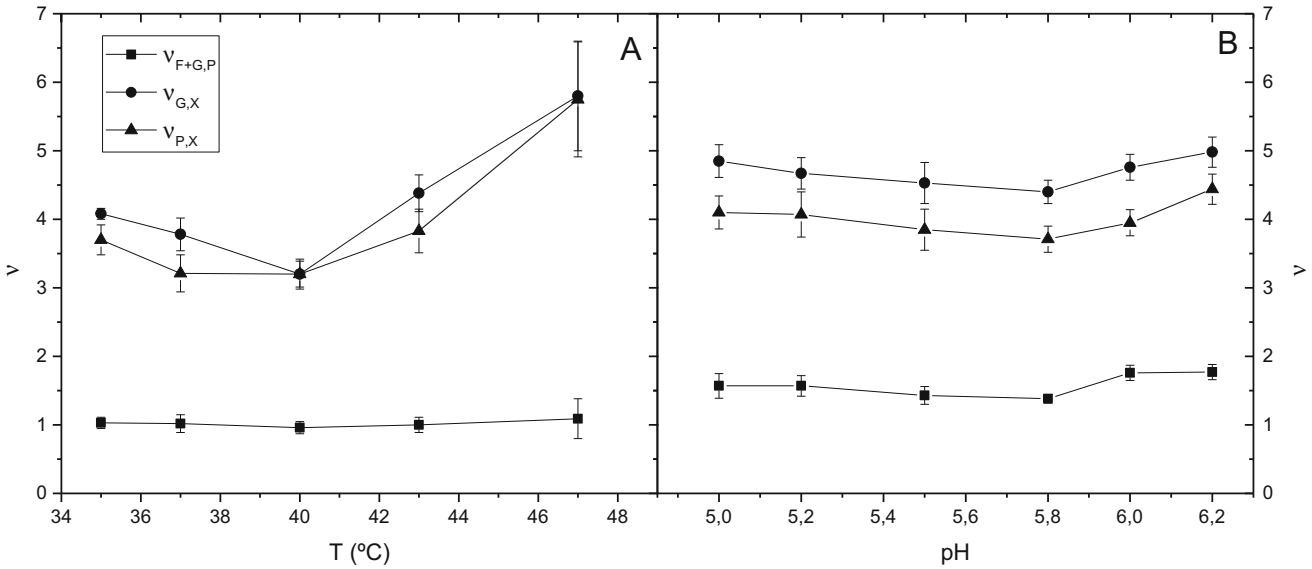

Fig. 3 Influence of temperature (**a**) and pH (**b**) on the values of the coefficients of the proposed kinetic model for the runs carried out to study the influence of these variables on D-LA production

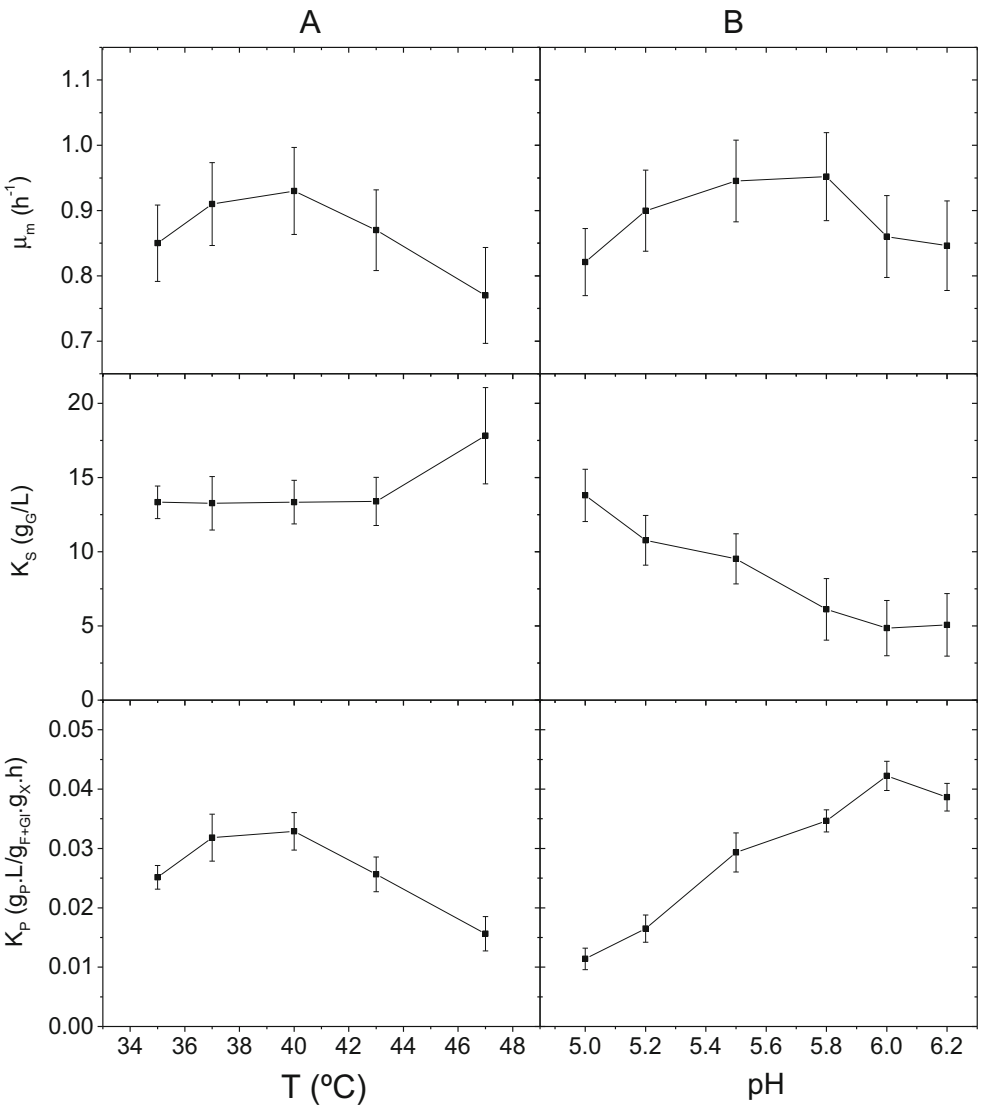

Fig. 4 Influence of temperature (**a**) and pH (**b**) on the kinetic parameters (μ_m, K_S, and K_P) of the proposed model

similar to that of glucose, with a progressive phosphorylation previous to its splitting into dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP) [37].

3.2 Kinetic modeling

The determination of the kinetic parameters and coefficients was performed by fitting the eqs. (7) to (12) to all the data (biomass, glucose, fructose + galactose, and D-lactic acid concentrations) obtained from the pH and temperature studies. Therefore, a set of parameters values is obtained for each run.

Table 2 shows the values obtained for the statistical parameters for the experiments at different temperatures and pH values. The kinetic model fits very remarkably to experimental data, as indicated by the values of the goodness-of-fit parameters in all cases: the root mean square error (RMSE) has a very low value in relation to average values of all concentrations, in particular to C_{DLA}. If Fisher's F parameter (F_{calc}) is taken into account, its value is much higher than F-Fisher tabulated at 95% confidence (F_{tab}) for all experiments, thus indicating that the null hypothesis is fulfilled and the model is statistically correct. A last statistical criterion is the percentage of variation explained (%VE), a criterion that focuses on the variation of dependent variables with time, the independent one. In this case, all the experimental trends are explained up to the 99% level with only one exception, which is acceptably explained (98%), reinforcing the validity of the proposed kinetic model in terms of goodness of fit. This is clearly shown in Figs. 1 and 2, where plotted lines are the model predictions and points are experimental data.

The values of the model coefficients ($\nu_{G,X}$, $\nu_{P,X}$, and $\nu_{F+Gl,P}$) obtained by fitting are displayed in Fig. 3, indicating the influence of temperature in Fig. 3a and the effect of pH in Fig. 3b. In particular, the relative consumption of glucose per gram biomass ($\nu_{G,X}$) and the relative production of lactic acid per gram biomass ($\nu_{P,X}$) is mostly affected by temperature: both

present a minimum value at 40 °C, where 1 mol of product is obtained out of 1 mol of glucose or the fructose + galactose mixture, being this the best ratio product/substrate achieved in this work. However, pH scarcely influences these parameters. The coefficient $\nu_{F+Gl,P}$ is affected neither by temperature nor pH, indicating that these process variables scarcely impact the consumption of fructose and galactose by the bacterium. Thus, when there is still glucose in the broth and the reaction involves not only production but also growth, the carbon flux seems to be influenced by temperature, but not by pH.

Regarding the kinetic parameters (μ_m, K_s, and K_p) of the model, Fig. 4 shows the trends of all of them with temperature (Fig. 4a) and pH (Fig. 4b). The only parameter that shows similar trends for temperature and pH is the maximum growth rate (μ_m). Its value is maximal at 40 °C and pH 5.8, showing the best ranges of temperature and pH for the growth. Regarding the influence of the variables on K_S, it is observed

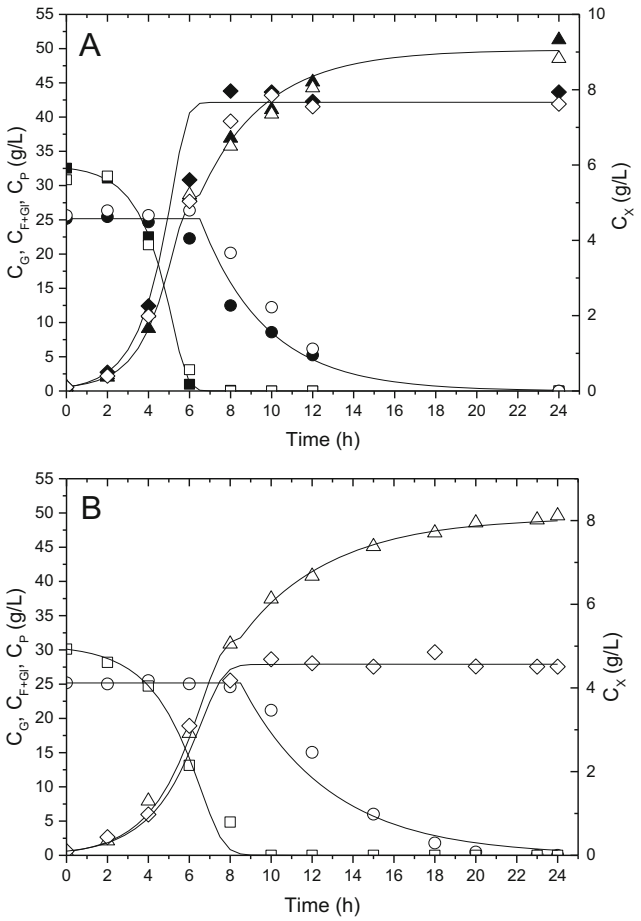

Fig. 5 Comparison of the experimental results obtained from simulated and real OPW hydrolysate and time course prediction by the proposed kinetic model. Data points: glucose (squares), fructose + galactose (circles), lactic acid (triangles), biomass (diamonds). Closed symbols are the experimental results obtained from the simulated OPW hydrolysate and open symbols are those ones from the real OPWH, both with **a** MRS and with **b** CSL nitrogen sources. The lines show the model prediction of all these runs

that it presents a constant value for almost the whole temperature range studied, with a notable increase for 47 °C, indicating a slightly lower affinity for glucose at this extreme temperature. However, when pH influence is studied, there is a decreasing trend for this parameter in the studied pH range, suggesting an increase in the affinity of the microorganism for glucose as pH rises.

The kinetic constant for the production from fructose + galactose (k_p) shows a parabolic trend with temperature, reaching a maximum value at 37 to 40 °C. In fact, 40 °C seems to be the best temperature for both parts of the process (glucose or fructose + galactose consuming stages) in terms of productivity. Regarding the influence of pH in the second part of process, k_p present maximum values at pH values higher than 5.8. This is not surprising, as the highest productivities are obtained in the same pH range (Table 1). *Lactobacillus* is a genus of LAB particularly resistant to low pH values, able to withstand external pH values as low as 3.5, using H^+-coupled ion transport systems to maintain a more alkaline cytoplasmic pH. Threshold pH values depend on genus and species, but set a lower limit to bacterial growth and metabolism, affecting all production pathways, in particular those that reduces internal pH, like the production of organic acids. Considering best tested pH for *Lactobacillus*, it can be even lower than 5.7, probably due to the efficiency of proton pumping by H^+-ATPase [38].

In short, temperatures from 37 to 40 °C and pH equal or slightly higher than 5.8 are the best conditions for the LAB growth and D-lactic acid production, while higher pH values are better for D-LA production out of fructose + galactose, but worse for the growth and D-LA production when glucose is the substrate (at the beginning of the process).

3.3 D-lactic acid production from real orange peel waste hydrolysates

Once the kinetic parameters were estimated by fitting the proposed model to all data (run by run), knowing the best values

Table 3 Parameter values obtained from the fit of the proposed kinetic model to data from run 9 (model OPWH supplemented with MRS components other than sugars) and run 12 (real OPWH with MRS components but glucose), and run 13 (real OPWH supplemented with 37 g/L CSL)

Parameter (unit)	Run 9 and 12	Run 13
μ_m (h^{-1})	0.95 ± 0.07	0.67 ± 0.05
K_S (g_G/L)	6.44 ± 0.95	6.91 ± 1.05
k_P (g_PL)/(g_{F+G}.g_X·h)	0.036 ± 0.003	0.032 ± 0.004
$\nu_{P,X}$ (g_P/g_X)	3.81 ± 0.42	7.47 ± 0.66
$\nu_{G,X}$ (g_G/g_X)	4.63 ± 0.37	7.06 ± 0.58
$\nu_{F+Gl,P}$ (g_{F+G}/g_P)	1.02 ± 0.06	1.40 ± 0.13

of pH (5.8) and temperature (40 °C), the model was validated with data from a run that was carried out at the best pH and temperature values tested using as substrate a real orange peel hydrolysate. As in the model experiments, the hydrolysate was supplemented with most nutrients present in the MRS medium (nitrogen sources and other components except glucose). This run was performed in a 2-L bioreactor using identical conditions as in the best experiment performed with the model mixture of monosaccharides (run 9). In this case, the kinetic model was used to simulate the concentration evolution of carbon substrates, biomass, and lactic acid using the values of the parameters obtained for run 9. As it can be seen in Fig. 5a, the model is able to simulate very closely the temporal evolution of the concentrations of all components in this run performed using a real OPWH supplemented with MRS components. This is not surprising, as little difference is observed in experimental data between run 9 (model mixture of monosaccharides resembling OPW hydrolysate) and run 12 (the real OPW hydrolysate). Therefore, compounds such as terpenes and phenolics common to this type of hydrolysate scarcely affect LAB activity in terms of lactic acid production. What is more important, the results from model sugar mixtures seem to be enough to predict D-lactic acid production when using a real OPW hydrolysate.

As nitrogen sources present in MRS broth are very expensive from an industrial perspective, another run (run 13) to check the kinetic model was performed. In this case, nitrogen source was added using corn steep liquor (CSL) from Roquette (SOLULYS® 048E) to 37 g/L. The results of this run (titter, yield, productivity, and purity) are collected in Table 1. Figure 5b shows the temporal concentration evolution of all relevant components. If compared to Fig. 5a, biomass growth is slower and its maximal concentration (in the stationary growth phase) is lower. However, the concentrations of the other components (in particular, lactic acid) are similar to the ones observed when using MRS medium, so the same kinetic model can be applied to this run in this regard. When fitting the model to data in run 13, very good values of the goodness-of-fit statistical parameters are obtained, as shown in Table 2. Kinetic parameters are collected in Table 3, together with those of run 9—for the sake of comparison—while the predicted data from the model are shown as lines in Fig. 5b, indicating that this model is adequate to describe the D-LA production process from OPWH supplemented with this CSL solution.

When comparing kinetic parameters for runs 9 and 13, it is evident that the bacterium is equally active, considering the reaction volume, to produce D-lactic acid. However, the values of the stoichiometric coefficients suggest that the specific activities are higher when CSL is used, as less biomass is able to produce an equal concentration of D-lactic acid and consume glucose, fructose, and galactose in the same time.

4 Conclusions

L. delbrueckii spp. *delbrueckii* produces D-lactic acid in two stages when using orange peel wastes hydrolysates or resembling sugar mixtures: firstly, glucose is used both for growth and production; secondly, fructose and galactose are consumed for production. This is reflected in a kinetic model that fits successfully to all experimental data, being able to predict the temporal concentration evolution of biomass, glucose, fructose-galactose mixture, and D-lactic acid. The model is accurate regardless of the temperature and pH value tested, for model sugar solutions and OPWH with MRS or CSL supplementation. The best tested values for the variables under study are 40 °C and pH 5.8, respectively, in terms of D-lactic acid production, biomass growth, and substrate uptake.

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Nomenclature C_j, Referred to concentrations of compound j (g_j/L).; D-LA, D-lactic acid.; F, Fischer-F, statistical parameter.; K_s, Saturation constant for the glucose (g_G/L).; k_P, Kinetic constant for D-lactic acid production from fructose and galactose (g_PL/g_{F+Gl}·g_X h).; M, Number of parameters of a proposed model.; N, Total number of experimental data used for fitting.; P, Productivity of D-lactic acid (g/L·h).; r_i, Reaction rate of the reaction i (g/L·h).; RMSE, Residual mean squared error.; SSQ_l, Sum of squared residues respect to the calculated value of "l" variable.; SSQ_{lmean}, Squared sum of deviations between the experimental and the mean score respect to The calculated values of the variable.; SSR, Sum of squared residuals.; t, Time (h).; VE, Variation explained of a nonlinear regression fitting (%).; $Y_{P,S}$, Yield of D-lactic acid referred to the total consumption of carbon sources (g/g).; y_i, Experimental or calculated values of "i" variables.

Greek letters μ_m, Maximum biomass growth rate (h^{-1}).; ν_{ij}, Coefficients in the reaction scheme (eqs. (3) and (4)) ($g_i \cdot g_j^{-1}$).

Subscripts A, Referred to arabinose.; calc, Referred to the calculated value of a variable from the model.; F, Referred to fructose.; G, Referred to glucose.; Gl, Referred to galactose.; P, Referred to product (D-lactic acid).; S, Referred to the sum of all the substrates.; tab, Referred to a F-value given by F-test tables for a confidence interval.; X, Referred to biomass.

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