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Optimization of phenyllactic acid production by *Pediococcus acidilactici* CRL 1753. Application of the formulated bio-preserver culture in bread

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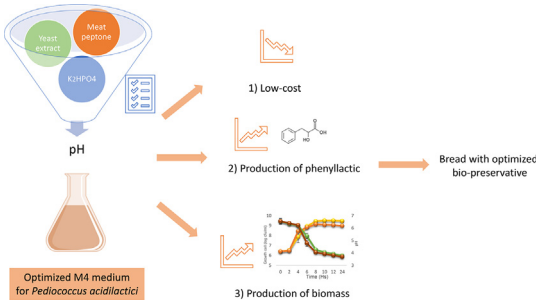
GRAPHICAL ABSTRACT



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ABSTRACT

Phenyl lactic acid (PLA) is an antimicrobial agent with a broad antimicrobial spectrum produced mainly by lactic acid bacteria. This study focused in the optimization of a low-cost medium to obtain biomass and PLA production by *Pediococcus (Ped.) acidilactici* CRL 1753 strain and propose its use as a liquid bio-preserver in bread making to increase the shelf life of wheat bread. First, the activity of CRL 1753 strain against filamentous fungi and yeasts was study and a wide antifungal inhibitory spectrum was observed. Using a factorial design 2^{9-5} an optimized and low-cost medium (10 g/L meat peptone, 5 g/L yeast extract, 10 g/L glucose, 0.0038 g/L $MnSO_4$, 6 g/L K_2HPO_4 and initial pH = 7.0) that could replace MRS was obtained. A significant increase in shelf life was observed when calcium propionate (CP) and CRL 1753 strain were combined into the bread formulation. In fact, at 18 days of storage none moulds were observed when the liquid bio-preserver was added while 70% of breads were spoilage with CP alone. In this work we report for the first time the obtaining of an optimized low-cost culture medium for biomass and PLA production by *Ped. acidilactici* strain. The results obtained could be very useful as a basis to design new strategies that allow improve the PLA production at a low cost.

1. Introduction

Spoilage of bread is a common event, frequently caused by fungal growth and origin considerable economic losses for bakery industry (Dewettinck et al., 2008). Calcium propionate (CP) is the chemical additive of choice to inhibit bread moulds (Ryan et al., 2008). However, recent studies showed that, at the maximum allowed concentrations (0.3% w/w in Europe, Directive and TO, 2000), CP was not effective against the most frequent deteriorating organisms (Lavermicocca et al., 2003).

The use of lactic acid bacteria (LAB) as bioprotective cultures is a common alternative to reduce the concentration of chemical

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preservatives to extended shelf life and enhanced safety of bakery products (Axel et al., 2016; Axel et al., 2017; Gerez et al., 2010). So far, among the best candidates, *Lactobacillus* (*L.*) strains are the most usually reported (Gerez et al., 2009; Karami et al., 2017; Mieszkin et al., 2017) highlighting the studies in *L. plantarum* (Deepthi et al., 2016; Gerez et al., 2014; Guimarães et al., 2018; Russo et al., 2017). In this sense, several reports showed that *Lactobacillus* are the dominant species in sourdough fermentation (Ventimiglia et al., 2015; Dolci and Cocolin, 2017; Gobbetti et al., 2016) while Corsetti et al. (2007) stated that *Pediococcus (Ped.)* and *Enterococcus* appear as subdominant species in Italian sourdough. Co-fermentations between them, showed that subdominant LAB are stronger acidifiers than typical species (such as *L. sanfranciscensis*) at the beginning of sourdough production and might prepare the environment for the establishment of dominant species in develops sourdoughs. Other authors also reported the incorporation of *Ped.* strains as complementary microflora in sourdough preparation (Paramithiotis et al., 2005; Katina et al., 2002). In a more recent study autochthonous strains, including *Ped. acidilactici*, were isolated from hop (*Humulus lupulus*) and employed as starters culture for wheat bread making (Nionelli et al., 2018).

Between the antifungal metabolites produced by LAB, phenyl lactic acid (PLA) has been widely reported and studied. This new antimicrobial compound has a broad antimicrobial spectrum, greater stability and solubility and it has also been proposed as an indicator of the antifungal capacity of LAB (Juodeikiene et al., 2018; Dal Bello et al., 2007;). Production of PLA was reported by various LAB strains (Gerez et al., 2009; Valerio et al., 2004; Yu et al., 2014; Barman et al., 2017), however, the available information about this antifungal compound produced by strains of *Ped.* spp. and its use as bio-preservatives is still scarce (Mandal et al., 2013; Muhialdin et al., 2011; Sellamani et al., 2016). From a commercial point of view, the production of high concentrations of PLA for its use as a pure additive could be obtained using chemical methods; though the extreme condition reactions employed are not environmentally friendly (Axel et al., 2017). On the other hand, the commercialization of PLA-producing strains to use as starter is an alternative that can respond to the demands of consumers for more natural preservation systems. This latter option is restricted because of low production by the known producing strains (Schnürer and Magnusson, 2005). These limitations make it necessary to explore alternatives that allow improving the production of this metabolite of interest. In this sense, a useful strategy is the optimal design of a culture medium. The application of statistical experimental design techniques in fermentation allows to understand the interactions between the parameters of the process and, in this way, improve the performance of the product, reduce the variability of the process and reduce times and costs of development (Mu et al., 2009; Ren et al., 2008).

In previous study, *Ped. acidilactici* CRL 1753 showed the highest antifungal activity against common spoilage moulds between several lactic strains isolated from different sources (data not published). The inhibitory effect was attributed mainly to the production of PLA. The aim of this work was to optimize a low-cost medium to improve biomass and PLA production by *Ped. acidilactici* CRL 1753. Furthermore, the liquid bio-preserver CRL 1753 was evaluated in bread making to increase the shelf life of wheat bread.

2. Materials and methods

2.1. Microorganisms and culture conditions

Ped. acidilactici CRL 1753 isolated from silage and belonging to the Culture Collection of the Centro de Referencia para Lactobacilos (CERELA-CONICET), Tucumán was used in this study. In a previous screening this strain showed the maximum antifungal activity and PLA production among several lactic bacteria strains isolated from different sources, so it was selected for this work (unpublished data). Lactic culture was grown in MRS (De Man et al., 1960) broth (pH 6.5) at 37 °C for 24 h. Before experimental use, cultures were propagated (1%, v/v) in MRS medium and incubated at 37 °C for 18 h without agitation.

The moulds strains used in this study were *Aspergillus* (*A.*) *niger* CH1, CH2 and CH3, *A. japonicus* CH5, *Penicillium (P.) sp.* CH6, *P. digitatum* CH10, *P. roqueforti* CH4, *Candida (C.) tropicalis* CH6, *Metschnikowia (M.) pulcherrima* CH7, *Pichia anómala* CH8, *Saccharomyces (S.) cerevisiae* CH9. All fungi strains were isolated from contaminated breads while the yeasts were isolated from fruit juice. The moulds strains were grown on potato dextrose agar medium (Britania, CABA, Argentina) at 25 °C for 7 days. The conidias were collected in sterile Tween 80 at 0.05% (v/v), counted in Buerkner hemocytometer, and adjusted to 10^5 per ml of sterile water (0.2%). Yeasts were grown in YPD medium (1.0% w/v yeast extract, 2.0% w/v peptone, 2.0% w/v glucose) at 30 °C for 24 h.

2.2. Antifungal assay

Antifungal activity of *Ped. acidilactici* CRL 1753 was determined by antifungal screening assay (Inglin et al., 2015) as described below. The assay was performed in a 24-well cell-culture plate (Greiner Bio One, Buenos Aires, Argentina) containing 500 µl of 1.5% (w/v) MRS agar. *Ped. acidilactici* CRL 1753 was spotted at the center of a well with 10 µl of an outgrown culture and incubated for 48 h. Thereafter wells were overlaid with 500 µl of 0.5% Sabouraud's glucose soft-agar (Britania, Argentina) and inoculated either with 10^3 fungal spores per ml or 10^4 yeast per ml. The plates were incubated for 24–48 h at optimum conditions for the indicator strain. All experiments were performed as duplicates. The inhibition areas were visually recorded daily. The results were expressed as: negative (absence of zone of inhibition), positive + + (zone of inhibition defined on LAB colony) and strongly positive + + + (absence of fungal growth in 100% of well). In addition, the antifungal activity of the CRL 1753 strain was evaluated in neutral condition. For this purpose, MRS agar and Sabouraud's glucose soft-agar media with 0.1 M de K_2HPO_4 pH = 7 were used. The organic acids production by *Ped. acidilactici* CRL 1753 was determined after 24 h fermentation in MRS medium by high-performance liquid chromatography (Gerez et al., 2014).

2.3. Optimization of biomass and PLA production

In order to study the influence of each component of the MRS medium on the production of biomass, a factorial design 2^{9-5} was used. It implies the evaluation of one faction of the total of possible treatments (culture media) resulting of the combination of 9 factors (components of MRS medium). The factors taken into consideration were: meat peptone, meat extract, yeast extract, K_2HPO_4, ammonium citrate, glucose, Tween 80, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$. A total of 20 combinations were assayed. The design combinations are presented in Table 2. The different culture media were inoculated (1%, w/w) with CRL 1753 strain and incubated 18 h at 37 °C. The responses evaluated were LAB growth (log CFU/ml) and PLA production by high-performance liquid chromatography (Gerez et al., 2014). The statistical analysis was performed considering the both responses in each medium tested. Desing-Expert 7.0 (Stat-ease) program was employed to predict the different combinations of the ten factors and the interactions among them.

2.4. Effects of K_2HPO_4 on PLA production

Fermentations were conducted in 200 ml of M4 broth, supplemented with K_2HPO_4 ranged between 2.0 and 12.0 (g/L). Medium without K_2HPO_4 was used as a control. Samples were taken at the end of the fermentation to determine the LAB growth and PLA concentration as previously described.

2.5. Effects of initial pH on PLA production

The effect of pH on the growth of strain and the production of PLA

was studied. The pH of M4 broth was adjusted to 5.0, 5.5, 6.0, 6.5 and 7.0 by adding sterile 5 N NaOH or 2 N HCl. All pH adjustments were done after autoclaving. Samples were taken at the end of the fermentation to determine the LAB growth, PLA concentration as previously described.

2.6. Ped. acidilactici CRL 1753 as starter for bread making

Ped. acidilactici CRL 1753 was inoculated (1%, v/v) in optimized M4 medium (10 g/L glucose, 5 g/L yeast extract, 10 g/L peptone, 0.38 g/L $MnSO_4$, 6 g/L K_2HPO_4, initial pH 7) and incubated at 37 °C during 24 or 48 h. These cultures ($CRL1753_{24h}$ and $CRL1753_{48h}$) were used for wheat bread manufacture. The dough (DS1753) was prepared as follows: 1000 g wheat flour, 15 g dried yeast (Levex, Santiago de Chile, Chile), 20 g NaCl, 30 g fat (baking margarine, Danica Dorada, CABA, Argentina), 30 g dried skim milk (Veronica, CABA, Argentina), 3 g CP (preservative commonly used in bread as an antifungal agent) and 0.55 L of tap water (Gerez et al. 2014, 2015). In the formulations with the liquid bio-preserver CRL 1753 consisting in lactic culture biomass and supernatant with antifungal metabolites), 35% (v/v) of tap water was replaced by equal amounts of the liquid culture. At the beginning of fermentation the number of LAB was about 7.0 log CFU/ml to both doughs with lactic culture. Doughs without lactic starter were used as control (Dc) with initial LAB count of 2.5 log CFU/ml). The doughs were individually placed in aluminum pans for fermentation (2h at 37 °C) and samples were taken at the end this period to determine pH (pH meter PT-10 model, Sartorius AG, Goettingen, Germany), microbial viability and organic acids as was previously reported (Gerez et al., 2014). After fermentation, the doughs were baked in an oven (180 °C). Cooked breads were cooled in a laminar flow cabinet to prevent contamination. The bread loaves were surface sprayed (1 ml per 100-g loaf) with a conidial suspension (10^3 conidia per ml) of *A. japonicus* CH5; then they were packed into polyethylene bags and stored at 30 °C. The bread shelf life was defined as the time (in days) for moulds to become visible on the surface of the packaged loaves. Observations were performed daily.

2.7. Statistical analysis

All assays were performed in three independent experiments and mean values ± standard deviation (SD) are given. Data were compared by analysis of variance (Anova) and Dunnett *t*-test. The statistical significance ($P < 0.05$) was determined by using InfoStat2006p.3 software. The principal component (PCA) and cluster analysis were applied to the LAB antifungal activity data.

3. Results

3.1. Antifungal spectra of Ped. acidilactici CRL 1753

In this work, the antifungal activity of *Ped. acidilactici* CRL 1753 against six filamentous fungi and four yeasts was studied. These moulds were chosen because they are the causative agents of frequent spoilages of a wide range of food matrices. The inhibitory activity was evaluated in two conditions; acidic and neutral pH. As is shown in Table 1, CRL 1754 strain had a broad antifungal inhibitory spectrum, with activity against all genera of moulds and on the yeast *M. pulcherrima* CH7. The antifungal effect on mould germination was not observed after neutralization indicating the acidic nature of the metabolites involved. The organic acids production by *Ped. acidilactici* CRL 1753 was determined after 18 h fermentation in MRS medium. Lactic (8.8 ± 0.45 g/L), acetic (0.18 ± 0.05 g/L) and PLA (117.7 ± 1.25 mg/L) acids were detected. Therefore, we set out to optimize the production of this metabolite together with the biomass of the lactic strain to propose a liquid bio-preserver starter (biomass and antifungal metabolites) to bread making.

Table 1
Inhibitory spectra of *Ped. acidilactici* CRL 1753.

Fungus	Inhibitory activity	
	Acidic condition[1]	Neutral condition[2]
A. niger CH1	−	ND
A. niger CH2	+ + +	−
A. niger CH3	−	ND
A. japonicas CH5	+ + +	−
P. roqueforti CH4	+ + +	−
P. digitatum CH10	+ + +	−
S. cerevisiae CH9	−	ND
M. pulcherrima CH7	+ + +	−
C. tropicalis CH6	−	ND
P. anómala CH8	−	ND

Negative (absence of zone of inhibition), positive + + (zone of inhibition defined on LAB colony), strongly positive + + + (absence of fungal growth in 100% of well). ND: not determined.
[1] Antifungal activity at acidic pH.
[2] Antifungal activity at neutral pH.

3.2. Optimization of biomass and PLA production

The first step for searching for the optimal conditions was to identify the variables that are of a significant influence on the desired response (biomass and PLA production). In this work, the nine components of MRS medium were set as nine different variables. The 16 experiments and four other experiments at the center of the design were added for analysis of the factorial design required variances. The experimental design and the results are illustrated in Table 2.

The goodness of fit of the model was verified by the determination coefficient (R^2). In this study, the R^2 value for biomass and PLA production was 0.9762 and 0.9628, respectively, which indicates that most of the variability in the responses is explained by the proposed model (Table 3). The Model F-values (50.121 and 32.07) implies the model is significant. There is only a 0.40 and 0.77% chance that a "Model F-Value" this large could occur due to noise.

The experimental design allowed establishing the components of the culture medium that have a significant effect on the evaluated responses (biomass-PLA production). Seven components of the medium had a significant effect ($P < 0.05$) on the growth of the strain, being meat peptone, meat extract and $MnSO_4$ the components that showed positive main effects. Otherwise, PLA production, increased with an increase in the concentration of meat peptone, meat extract and K_2HPO_4. Some interactions between the components of the culture medium also showed a significant effect on the parameters evaluated. Some two-component interaction effects are explained graphically in Supplementary material 1 and 2. In the presence of 10 g/L meat peptone, the addition of yeast extract from 2.5 to 5 g/L increased the growth of the lactic strain (Supplementary material 1a). On the other hand, in presence of 10 g/L meat peptone, supplemented with meat extract from 0 to 10 g/L did not modify PLA production (Supplementary material 1b). According to significant effect and interactions, the composition of the culture medium, named M4 medium was: 10 g/L meat peptone, 5 g/L yeast extract, 10 g/L glucose, 0.0038 g/L $MnSO_4$, and 2 g/L K_2HPO_4.

3.3. Effects of K_2HPO_4 and initial pH on PLA production

Considering that the presence of K_2HPO_4 in the culture medium positively affected the PLA production, we studied the effect of different concentrations of this compound on the growth of CRL 1753 strain and PLA yielding. The maximum concentration of K_2HPO_4 that was soluble in the experimental medium was 12 g/L. As shown in Fig. 1a, the addition of 2–6 g/L of K_2HPO_4 resulted in a significant increase of PLA production reaching the maximum value of 133.73 mg/L after 18 h of

Table 2
Experimental design and results of the factorial design 2^{9-5}.

Run	Independent variables (g/L)									Response function	
	Meat peptone	Meat extract	Yeast extract	$MnSO_4$	$MgSO_4$	Ammonium citrate	K_2HPO_4	Glucose	Tween 80 (ml)	Biomass (log CFU/ml)	PLA (mg/L)
1	10	10	2.5	0.038	0	0	0	20	0	9.1	77.4
2	0	0	2.5	0.038	0	2	2	20	0	8.8	46.8
3	0	10	5	0.038	0	2	0	10	0	8.9	66.2
4	10	0	5	0.038	0	0	2	10	0	9.2	119.5
5	10	10	5	0	0.05	0	0	10	0	9.7	92.2
6	0	10	2.5	0	0.05	2	0	20	0	8.5	48.6
7	0	10	5	0	0	0	2	20	1	8.5	95.4
8	5	5	3.75	0.019	0.025	1	1	15	0.5	9.2	92.7
9	10	10	2.5	0	0	2	2	10	1	8.6	127.7
10	0	0	5	0.038	0.05	0	0	20	1	8.2	25.4
11	0	0	2.5	0	0	0	0	10	1	7.5	4.0
12	10	0	2.5	0	0.05	0	2	20	0	8.3	117.9
13	5	5	3.75	0.019	0.025	1	1	15	0.5	9.2	87.7
14	10	0	2.5	0.038	0.05	2	0	10	1	8.9	88.9
15	10	0	5	0	0	2	0	20	1	8.6	83.3
16	5	5	3.75	0.019	0.025	1	1	15	0.5	9.0	102.5
17	0	10	2.5	0.038	0.05	0	2	10	1	9.3	98.3
18	0	0	5	0	0.05	2	2	10	0	8.2	86.4
19	5	5	3.75	0.019	0.025	1	1	15	0.5	9.1	96.2
20	10	10	5	0.038	0.05	2	2	20	1	9.5	117.9

fermentation at an initial concentration of 6 g/L. However, at higher concentrations of phosphate, the PLA concentration decreased.

The effect of initial pH on growth and PLA production is shown in Fig. 1b. In this assay, M4 medium was supplemented with 6 g/L K_2HPO_4. Changes in pH did not significantly modify the growth of CRL 1753 strain. However, the production of PLA was strongly dependent on the initial pH of medium reaching the maximum yield of 148.60 mg/L at pH 7. In consequence, a new optimized M4 medium was obtained. To validate this result, *Ped. acidilactici* CRL 1753 was cultured both in MRS and M4 media for 24 h. Different fermentation parameters (growth, pH and PLA production) were evaluated during incubation. *Ped. acidilactici* CRL 1753 growth (9.2 Log CFU/ml) in M4 medium after 18 h, was like that observed in MRS medium (9.5 Log CFU/ml) while no significant difference about PLA production was observed, reaching a value of 157.84 mg/L at 24 h of incubation (Fig. 2). Thus, optimized M4 could replace MRS and its cost was only 4.24 USD/L compared with 9.27 USD/L of MRS.

3.4. *Ped. acidilactici* CRL 1753 as started in bread making

In order to obtain a liquid bio-preserver starter (biomass and antifungal metabolites) to bread making, the CRL 1753 strain was growing during 24 or 48 h in optimized M4 broth and subsequently these cultures ($CRL1753_{24h}$ and $CRL1753_{48h}$) were used for wheat bread manufacture. The fermentation time was extended (regarding above assays) to increase the PLA concentration. An increase of biomass (3.3 log CFU/ml), a marked decrease of pH (6.9 to 3.8) was observed after 24 h of incubation while no important differences were observed at the end of fermentation (48 h). In fact, increased of cell counts from 9.38 to 9.64 log CFU/ml and pH values (pH 3.6) were obtained in 48 h culture. In the 24-hour culture, the concentrations of organic acids were: lactic (9.8 g/L), acetic (0.25 g/L) and PLA (186.5 mg/L), while at 48 h its concentrations increased (lactic acid: 12.95 g/L, acetic acid: 0.32 g/L, PLA: 196.4 mg/L). The chemical and microbiological characteristics of the doughs obtained with the culture of the lactic starter incubated at 24 and 48 h of incubation ($DS1753_{24h}$ and $DS1753_{48h}$) are summarized in Table 4. The $DS1753_{24h}$ and $DS1753_{48h}$ doughs had a higher LAB

Table 3
Regression analysis (ANOVA) for the biomass and PLA production.

Source	Biomass			PLA production		
	Contribution (%)	F-value	p-value	Contribution (%)	F-value	p-value
Model		50.12	0.0040	–	32.07	0.0077
A-Meat peptone	21.98	164.74	0.0010	42.10	202.39	0.0008
B-Meat extract	25.34	190.81	0.0008	7.71	37.12	0.0089
C-Yeast extract	4.40	33.64	0.0102	1.97	9.50	0.0540
D-$MnSO_4$	21.98	164.73	0.0010	0.08	0.37	0.5871
E-$MgSO_4$	2.51	19.12	0.0221	1.03	4.96	0.1124
F-Ammonium citrate	0.13	0.99	0.3932	0.43	2.05	0.2480
G-K_2HPO_4	1.40	10.58	0.0474	35.26	169.71	0.0010
H-Glucose	0.86	6.37	0.0858	1.68	8.09	0.0654
J-Tween 80%	3.14	24.08	0.0162	0.07	0.33	0.6074
Interactions						
AB	0.57	4.25	0.1313	6.60	31.79	0.0110
AC	6.28	47.36	0.0063	1.88	9.06	0.0572
AD	1.03	7.71	0.0692	0.13	0.63	0.4866
AE	0.23	1.71	0.2826	0.47	2.26	0.2298
AG	9.42	70.20	0.0036	0.58	2.78	0.1941
AH	0.73	5.55	0.0999	0.02	0.082	0.7936
Curvature		76.01	0.0032	–	15.68	0.0288

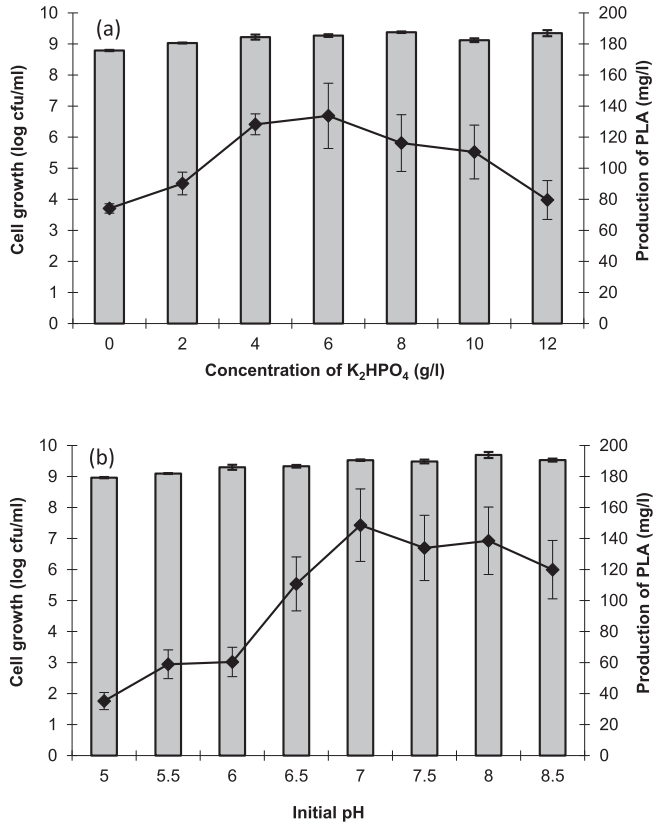

Fig. 1. Effects of K_2HPO_4 (a) and initial pH (b) on the growth and PLA production by *Ped. acidilactici* CRL 1753.

count (7.4–7.9 log CFU/g, respectively) and lower pH (5.0–5.1, respectively) than control dough (Dc) only made with commercial yeast. As was expected, PLA was only detected in doughs containing the lactic strain. Remarkably, lactic fermentation did not affect the activity of yeast, an aspect of great importance for the bakery industry as it does not affect the leavened of the dough or alter the baking process. Overall, the breads prepared with *Ped. acidilactici* CRL 1753 presented good organoleptic characteristics (data not shown).

Moreover, the ability of the two liquid bio-preservers CRL 1753 ($CRL1753_{24h}$ and $CRL1753_{48h}$) combined with traditional chemical additives to increase the shelf life of breads was evaluated. As shown in Fig. 3, cultures of CRL 1753 with 48 h incubation showed the highest antifungal effect. In fact, a significant increase in shelf life was observed when CRL 1753_{48h} culture and CP were combined into the bread formulation; at 18 days of storage none moulds were observed when the lactic strain was added while 70% of bread were spoilage in the bread made with CP alone.

Table 4
Characteristics of doughs made with *Ped. acidilactici* CRL 1753 (DS1753) or without (Dc) the addition of bio-preserver after fermentation at 37 °C.

Parameters	Dc	$DS1753_{24h}$	$DS1753_{48h}$
LAB colony counts (log CFU/ml)	3.3 ± 0.2	7.4 ± 0.3	7.9 ± 0.5
Yeast colony counts (log CFU/ml)	7.1 ± 0.1	7.23 ± 0.2	7.8 ± 0.6
Final pH	5.4 ± 0.2	5.1 ± 0.1	4.9 ± 0.1
Lactic acid (mM/Kg)	ND	5.5 ± 0.4	9.3 ± 0.12
Acetic acid (mM/Kg)	ND	ND	ND
Phenyllactic acid (mM/Kg)	ND	0.072 ± 0.005	0.083 ± 0.008
Propionic acid (mM/Kg)	19.8 ± 0.5	19.7 ± 0.2	19.5 ± 0.2

Dc: Control dough made from commercial yeast and calcium propionate (3 g/kg flour); $DS1753_{24h}$: dough made with lactic culture 24 h of incubation, commercial yeast and calcium propionate (3 g/kg flour); $DS1753_{48h}$: dough made with lactic culture 48 h of incubation, commercial yeast and calcium propionate (3 g/kg flour); ND: not detectable.

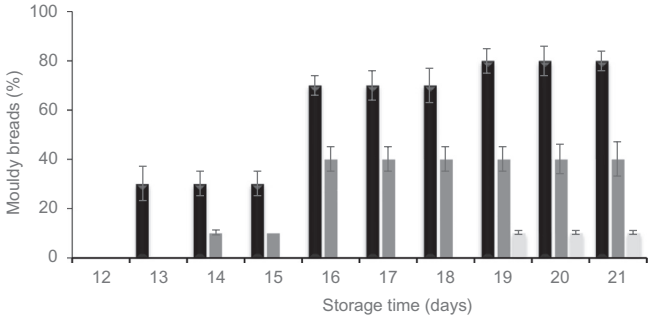

Fig. 3. Shelf life of packed brad storage at 30 °C.

4. Discussion

The antifungal activity is extensively studied in LAB, however most of the reports are focused in the use of *Lactobacillus* strains and very few studies tested *Pediococcus* strains (Sellamani et al., 2016; Karami et al., 2017; Shah et al., 2017a). In this work, we studied the antifungal activity of *Ped. acidilactici* CRL 1753 isolated from silage as other of its kind (Shah et al., 2017b). Our results showed that CRL 1753 strain produced a prominent inhibition zone against different fungal species. On the contrary, in a recent study, Lačanin et al. (2017) reported that none of 23 *Pediococcus* spp. strains tested showed antifungal activity against three spoilage fungi species. Similar findings were stated by Gerez et al. (2009) since three tested *Ped. acidilactici* strains displayed no antifungal activity against main fungal contaminants of breads. Although, *Ped. acidilactici* LAB 5 was found to have a broad-spectrum antifungal activity, however, active metabolite is an unknown compound of 83 kDa (Mandal et al., 2013). In this study, the antifungal effect of CRL 1753 strain on moulds germination was related with

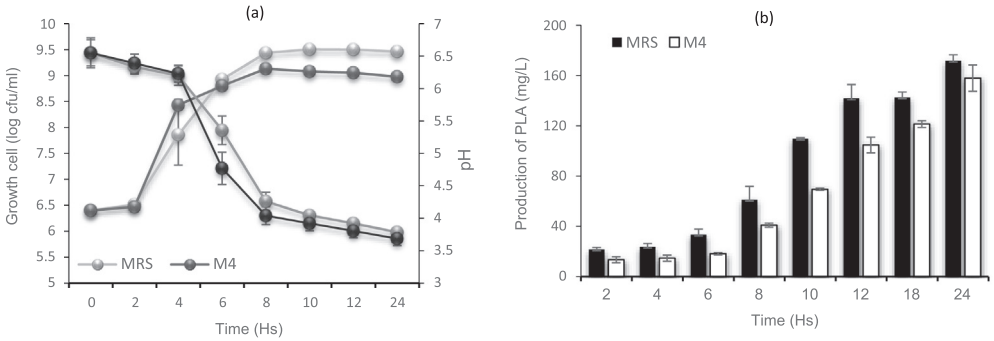

Fig. 2. Growth parameters (a) and PLA production (b) of *Ped. acidilactici* CRL 1753cultured in MRS and M4 media.

lactic, acetic and PLA. The latter organic acids have been described as an antimicrobial agent with broad-spectrum activity against bacterial and fungal pathogens (Lavermicocca et al., 2003; Zhang et al., 2014). Remarkable, antifungal activity of PLA was demonstrated against several species isolated from bakery products, including some mycotoxigenic strains (Cortes-Zavaleta et al., 2014; Gerez et al., 2014; Valerio et al., 2016). One of the main limitations of the use of PLA as food preservative is its low yielding by producing strains and the high costs of the process. Considering the above the purpose of the first optimization step was to identify which ingredient(s) of the growing medium (MRS) has a significant effect on biomass and PLA production by *Ped. acidilactici* CRL 1753. Our results showed that meat peptone, meat extract and $MnSO_4$ had significant effect ($P < 0.05$) on the growth while in the presence of meat peptone, meat extract and K_2HPO_4 higher PLA yielding were observed. In LAB, PLA is a product of catabolism of phenylalanine (Phe) in which the aminoacid is transaminated to phenylpyruvic acid (PPA), which is subsequently reduced to PLA (Yu et al., 2012). In this sense, it was reported that the addition of Phe or PPA to culture medium (Li et al., 2007; Rodríguez et al., 2012; Valerio et al., 2016; Valerio et al., 2004; Zhang et al., 2014) and citrate to wheat semiliquid ferment (Dallagnol et al., 2015) improved PLA production. These findings could explain the positive effect observed in the presence of meat peptone and yeast extracts since these are supplement rich in various nutrients specially vitamins, peptides and amino acids.

The concentration of K_2HPO_4 strongly affected PLA production without effect on cell growth suggesting that higher biomass could not be necessary related with higher yield of the organic acid. Concentration of PLA increased with an increase of K_2HPO_4 until reaching a maximum yield of 133.73 ± 21 at 6 g/L after which there was a decrease (Fig. 1a). The role of this compound in the yield of PLA was not previously established, however positive effects could be attributed to buffering capacity of K_2HPO_4, considering that initial pH had an important influence on the PLA production by CRL 1753 strain (Fig. 1). On the contrary, Mu et al. (2009) reported that addition of 3 g/L of K_2HPO_4 did not significantly affect the production of PLA by *Lactobacillus* sp. SK007 in an optimized culture medium. It was previously reported that in some LAB, K_2HPO_4 improve cell growth and synthesis of primary metabolites, such as nisin, in a dependent concentration way (De Vuyst and Vandamme, 1993; Von Mollendorff et al., 2009). In our work potassium phosphate did not affect growth of CRL 1753 strain, so further investigations are needed to establish the role of this compound in the production of PLA.

Remarkably, unlike growth, the PLA production was strongly dependent on the initial pH of the medium. At low pH (5–6) marginal amount of PLA were detected while at pH = 7 and with 6 g/L K_2HPO_4) the maximum yield of 148.6 mg/L was reached (Fig. 1b). Last metabolic step in the metabolic pathway of PLA is the reduction of PPA and is carried out by dehydrogenases where lactate dehydrogenase (LDH) is the most important. It was previously reported that optimum pH for pyruvate reduction by D(-) lactate dehydrogenase (LDH) of *Ped. cerevisiae* was at pH 8.0 and 3.6 at 5.0 and 0.5 mM of pyruvate, respectively (Gordon and Doelle, 1975). Thus, the highest yielding of PLA at pH 7 could be related with the optimum pH of the desidrogenase of *Ped. acidilactici* CRL 1753. In comparison, LDH enzymes of *Ped. pentosaceus* ATCC 25745 (Yu et al., 2012) and *Ped. acidilactici* DSM20284 (Mu et al., 2012a) were found to produce PLA at an optimum pH of 5.5 in agreement with the pH range of 5.5–7.0 reported for most LAB dehydrogenases (Mu et al., 2012a).

Ped. acidilactici CRL 1753 growth and PLA production in optimized M4 medium, was similar to that observed in MRS, and its cost was only 4.24 USD/L. Diminish the cost of the culture medium increases the workability of the industrial production of PLA. The maximum PLA production obtained was almost 160 mg/L (0.95 mM) in optimized M4 medium at an initial pH of 7 (Fig. 2). Even when the yielding of PLA strongly varied among genera and strains, our results agree with previous reports. In fact, in MRS medium (72 h at 30 °C) most of *Lactobacillus* strains produced less than 0.57 mM PLA (approximately 99 mg/L) (Mu et al., 2012b; Zhang et al., 2014). Propionic acid bacteria, on the contrary, exhibited production of very small quantities of PLA (0.01–0.1 mM) (Lind et al., 2007). As was mentioned above Phe and PPA had remarkable effect on PLA production in LAB strains, being the last one the most effective. In this sense, Valerio et al. (2016) showed that three *Lactobacillus* strains produced PLA in range of 0.44–0.93 mM when PPA was added to a defined growth medium for 72 h at 37 °C. In optimized substrate feeding and pH-control fed-batch fermentation, with PPA addition, *Lactobacillus* sp. SK007 reached a maximum of 17.38 g/L (100 mM) (Mu et al., 2009). There is very little information available about influence of precursors or components of culture medium on PLA production in *Pediococcus* strains. Mu et al. (2012b) showed that Phe and PPA addition to MRS broth effectively improve the PLA yielding by *Ped. acidilactici* DSM 20284 (36 h at 30 °C), reaching 1.46 and 3.42 mM, respectively compared with the 0.25 and 0.65 mM obtained without supplement.

The use of Lactobacilli strains producing PLA as starters of sourdough allowed the increase in shelf life in the bakery industry (Gerez et al., 2009; Lavermicocca et al., 2003; Ryan et al., 2008; Axel et al., 2016; Barman et al., 2017; Russo et al., 2017). In contrast, other authors were unable to reproduce the antifungal effect of certain LAB strains in wheat bread (Rosenquist and Hansen, 1998). In this study, the antifungal activity of *Ped. acidilactici* CRL 1753 was also confirmed in wheat bread. The longest shelf life (18 days) was achieved for packaged bread using a combination of 0.3% (w/w) CP and bio-preserver CRL1753 ($DS1753_{48h}$) since no moulds were detected while 70% of breads were spoilage in the absence of the lactic strain. This value was greater than previously reported (12 days) for wheat bread inoculated with fungi strains using a mixture of 0.3% (w/w) of CP and sourdough fermented with antifungal LAB (Ryan et al., 2008; Gerez et al., 2010; Gerez et al., 2015). The authors attributed these results to a potential synergistic effect between the LAB strain and CP.

Very few studies tested potential of *Pediococcus* strains to avoid spoilage in backed products. Muhialdin et al. (2011) showed that *Ped. pentosaceus* Te010 was able to delay appearance of *Aspergillus* strains between 3 and 5 days compared with the control bread. The antifungal activity was attributed to a protein-like compound. Cizeikiene et al. (2013) also reported that sourdough bread made with *Ped. pentosaceus* KTU05-9 suppressed ropiness by *Bacillus subtilis* spores until 6 days storage at 23 °C. In the same work, three *Pediococcus* strains sprayed as single cell suspension on the bread surface were able to inhibit fungal growth until 8 days of storage at 15 °C. Antimicrobial substances also have proteinaceous nature.

In conclusion, we report the obtaining of a low-cost culture medium for biomass and PLA production by *Ped. acidilactici* CRL 1753 strain. Thus, optimized M4 could replace MRS and its cost was only 4.24 USD/L compared with 9.27 USD/L of MRS. The microbiological safety of packed bread was guaranteed for 18 days with the addition of the bio-preserver combined with traditional chemical additives. To our knowledge, this is the first work reporting the optimization of culture medium components for PLA production by *Ped. acidilactici* strain and its effective use as bio-preserver. The results obtained could be very useful as a basis to design new strategies that allow improve the PLA production at a low cost.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.biocontrol.2018.05.017.

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