Research Article

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Valorization of orange peels exploiting fungal solid-state and lacto-fermentation

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Abstract

Background: Orange peels can serve as a cost-effective raw material for the production of lactic acid. Indeed, given their high concentration of carbohydrates and low content of lignin, they represent an important source of fermentable sugars, recoverable after a hydrolytic step.

Results: In the present article, the fermented solid, obtained after 5 days of *Aspergillus awamori* growth, was used as the only source of enzymes, mainly composed of xylanase (40.6 IU g^{-1} of dried washed orange peels) and exo-polygalacturonase (16.3 IU g^{-1} of dried washed orange peels) activities. After the hydrolysis, the highest concentration of reducing sugars (24.4 g L^{-1}) was achieved with 20% fermented and 80% non-fermented orange peels. The hydrolysate was fermented with three lactic acid bacteria strains (*Lacticaseibacillus casei* 2246 and 2240 and *Lacticaseibacillus rhamnosus* 1019) which demonstrated good growth ability. The yeast extract supplementation increased the lactic acid production rate and yield. Overall, *L. casei* 2246 produced the highest concentration of lactic acid in mono-culture.

Conclusion: To the best of our knowledge this is the first study exploiting orange peels as low-cost raw material for the production of lactic acid avoiding the employment of commercial enzymes. The enzymes necessary for the hydrolyses were directly produced during *A. awamori* fermentation and the reducing sugars obtained were fermented for lactic acid production. Despite this preliminary work carried out to study the feasibility of this approach, the concentrations of reducing sugars and lactic acid produced were encouraging, leaving open the possibility of other studies for the optimization of the strategy proposed here.

Keywords: lactic acid; orange peels fermentation; *Aspergillus awamori*; lactic acid bacteria

INTRODUCTION

Lactic acid (LA) is one of the most versatile molecules of industrial interest (food, cosmetic, pharmaceutical, chemical and medical industries) because it is an active precursor of different compounds like pyruvic acid, acetaldehyde or polylactic acid.[1-3] While its chemical synthesis gives a racemic mixture, microbial fermentation gives pure optical LA, which is essential for specific applications such as the production of polylactic acid.[4] Despite the high demand for this product, the cost of its production is a critical point, and novel strategies and more cost-effective raw materials are required to make feasible its biotechnological production.[1,4]

The citrus industry generates a substantial amount of waste and by-products which can be valorized, minimizing their disposal and, consequently, their environmental impact.[5] A large percentage of citrus fruit is consumed as processed products, especially as juices (1.7 million tonnes)[6] leaving peels, seeds and pulp as wastes, which represent approximately 45–60% of the entire fruit weight.[7] Among citrus fruits, orange is the most cultivated, representing around 50% to 60% of the total production, being 76 million tonnes in 2019.[8] The European Union produces approximately one-tenth of this amount, being Spain and Italy the main producers (80% of total production).[6]

Fermentation represents one of the most widely applied processes for the valorization of residues from the citrus industry[6,9-12] 12 and lactic acid bacteria (LAB) have been widely used for their capacity of growing in the presence of stressful compounds (such as polyphenols) and at low pH.[13-16] They can convert fermentable

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sugars from these residues into different compounds, especially LA.[5,17] Citrus residues must be previously hydrolysed for obtaining fermentable sugars. An enzymatic cocktail containing hydrolases, such as, cellulases, xylanases and pectinases is generally used. These enzymes can be produced through the fungal solid-state fermentation (SSF) of different matrices.[10,18] However, a subsequent extraction step of the enzymes is required and, in some cases, a purification stage[19,20] with a high impact on the total cost.[21]

Hydrolases are extensively used in several industrial sectors of the biotechnology field and they represent nearly 75% of the enzymes produced by this purpose. Among these sectors, the use for biofuels processes represents the largest proportion of the market of hydrolases, being Novozymes and DuPont the most important producers.[22,23] These enzymes are used for the hydrolysis of lignocellulosic components to obtain fermentable hexoses and pentoses.[24] To this purpose, the use of a single organism to produce the enzymes required could potentially be considered the most cost-effective process as it has lower capital costs.[25,26]

The aim of this study was the exploitation of orange peels (OPs) as a cost-effective raw material for the production of LA, avoiding the employment of commercial enzymes, which represent the major economical bottleneck of the process. For this purpose, in the first step OPs were fermented in a solid state (SSF) by using the filamentous fungus *Aspergillus awamori*. In the second step, the fermented solid was used as a source of enzymes for the hydrolysis of more OPs, releasing fermentable sugars which were finally metabolized by different LAB strains in mono- and co-cultures to LA. To the best of our knowledge, only a few authors have proposed this approach[19,20] but it has not been applied to the fermentation of OPs for the production of LA.

MATERIAL AND METHODS

Raw material conditioning

OPs (Washington Navel variety) were collected in the canteen of the University of Cadiz after juice extraction. For solid-state fermentations with *A. awamori*, washed orange peels (WOPs) were prepared as follows: initially, they were cut in pieces, milled in a blender, extensively washed with tap water to remove all water-soluble compounds including sugars and dried for 5 days at 40 °C until complete dehydration. After that, they were milled again to reduce their particle size. For enzymatic hydrolysis, OPs were cut into pieces, dried for 5 days in an oven at 40 °C, until complete dehydration, and finally milled to reduce their particle size.[6] Particle size distribution analysis of OP and WOP showed that 62.8% of the weight was constituted by particles over 1 mm in diameter.

Fibre analysis

Detergent fibre analysis of OPs and WOPs was carried out. Acid detergent fibre and acid detergent lignin were determined following EN ISO 13906:2008 method, whereas the amylase-treated neutral detergent fibre (NDF) method was selected for the determination of NDF (AOAC 2002:04/ISO 16472:2006). Based on these methods, different fractions can be quantified: (i) removable with acetone (fats, oils, etc.); (ii) removable with neutral detergent (proteins, enzymes, pectins, etc.); (iii) removable non-calcined (soluble salts); (iv) removable calcined (rest of extractable material but not saline); (v) removable with acid detergent (hemicellulose); (vi) extractable with concentrated acid (cellulose and soluble lignin); (vii) non-removable but calcined (insoluble lignin); (viii) non-removable and non-calcined (insoluble salts); and (ix) fraction not totally calcined (total salts).

Reducing sugars concentration analysis

Reducing sugars were measured by the dinitrosalicylic acid (DNS) method adapted for microplates.[27]

Solid-state fermentations

Spore propagation

Aspergillus awamori 2B.361 U2/1, a sequential mutant of *Aspergillus niger* NRRL 3312, was employed in this study (ABM Chemicals, Woodley, UK). The strain was stored at 4 °C on slants of a synthetic medium composed of 1 g L^{-1} peptone, 0.5 g L^{-1} yeast extract, 15 g L^{-1} agar, 6 g L^{-1} xylan, 5 g L^{-1} avicel and 1 g L^{-1} pectin. Spores stored on slants were washed with 2 mL of 0.9% *w/v* sodium chloride (NaCl), and 0.2 mL of this spore solution was spread on the surface of the same agar medium in Petri dishes and incubated at 30 °C for 5 days. After the incubation period, the spores were suspended in 1 mL of 0.9% *w/v* NaCl solution by gentle shaking. The number of the spores in the suspension was determined using an improved Neubauer counting chamber.

Solid-state fermentation (SSF)

SSF was performed in Petri dishes by adding 2 g of dried WOP and the required volume of a nutrient solution containing 2.4 g L^{-1} urea, 9.8 g L^{-1} $(NH_4)_2SO_4$, 5.0 g L^{-1} KH_2PO_4, 0.001 g L^{-1} $FeSO_4{\cdot}7H_2O$, 0.0008 g L^{-1} $ZnSO_4{\cdot}7H_2O$, 0.004 g L^{-1} $MgSO_4{\cdot}7H_2O$ and 0.001 g L^{-1} $CuSO_4{\cdot}5H_2O$ to adjust the initial moisture content of the solid to 6%. WOP and the nutrient solution were previously sterilized separately in an autoclave (121 °C for 20 min). The Petri dishes were inoculated with 7 log spore g^{-1} of *A. awamori* incubating them for 5 days at 30 °C in static conditions. OPs were washed before SSF to reduce their content of free sugars that otherwise would decrease the secretion of hydrolytic enzymes during the fungus growth. Thus, WOP was used as a solid substrate to induce the production of a wide variety of hydrolytic enzymes. The fermented WOP obtained was used immediately after its production as the only source of hydrolytic enzymes in the subsequent hydrolysis experiments.

Enzyme activities in the fermented orange peels (OPs)

To determine the enzyme activities contained in the fermented WOP an extraction with a Tween 80 solution was carried out. For this purpose, after 5 days of SSF, the whole content of each Petri dish was added to a 250 mL Erlenmeyer flask containing 20 mL of a 0.1% (*v/v*) Tween 80 solution. The suspension was incubated at 4 °C and 150 rpm for 30 min in a rotary shaker. Afterward, the suspension was centrifuged for 10 min at 10 000 rpm and 4 °C, and the supernatant liquor, the enzymatic extract, was stored at −80 °C until use.

Cellulase (FPase, EC 3.2.1.91), xylanase (EC 3.2.1.8), exo-polygalacturonase (EC 3.2.1.67), a type of pectinase, and β-glucosidase (EC 3.2.1.21) were measured in the enzymatic extracts. Cellulase assay was carried out by incubating 0.5 mL of diluted enzyme extract with 1 mL of citrate buffer (0.05 mol L^{-1}, pH 4.8), containing a Whatman No.1 filter paper strip (1 cm × 6 cm, 50 mg), at 50 °C for 60 min. For xylanase analysis, a mixture of 0.1 mL of diluted enzymatic extract and 0.9 mL of xylan suspension (0.5% *w/v* Birchwood xylan in 0.1 mol L^{-1} acetate buffer, pH 5.0) was incubated at 50 °C for 10 min. For exo-polygalacturonase activity, a reaction mixture containing 0.2 mL of diluted enzymatic extract and 0.8 mL of pectin suspension

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(0.5% *w/v* in 0.1 mol L^{-1} acetate buffer, pH 5) was incubated at 45 °C for 10 min. Reducing sugars produced after all these reactions were measured by the DNS method. A unit of enzyme activity (U) was defined as the amount of enzyme that produces 1 µmol of reducing sugars per 1 min under the specified conditions of pH and temperature. *β*-Glucosidase activity was determined by incubating 0.250 mL of 4 mmol L^{-1} *p*-nitrophenyl *β*-D-glucopyranoside (p-NPG) and 0.250 mL of acetate buffer (0.1 mol L^{-1}, pH 5) with 0.050 mL of enzymatic extract. The assay was performed at 60 °C for 10 min, after which 2 mL of sodium carbonate (Na_2CO_3) 2 mol L^{-1} were added to stop the reaction and allow the development of the yellow colour of the *p*-nitrophenolate ion, which was measured at 410 nm. *β*-Glucosidase activity was calculated based on the calibration curve of 4-nitrophenol. One unit of enzyme activity was defined as 1 µmol 4-nitrophenol equivalent released per minute.[28] All the experiments were conducted in triplicate.

Enzymatic hydrolysis of orange peels (OPs)

The enzymatic hydrolysis of OP was carried out by using fermented WOP as the only source of hydrolytic enzymes. For this purpose, 10 g of total solid, containing 20% (*w/w*) or 50% (*w/w*) of fermented WOP and 80% (*w/w*) or 50% (*w/w*) of the sterilized OP, respectively, were added to 175 mL citrate–phosphate buffer (pH 5.0, 0.05 mol L^{-1}) in 250 mL Erlenmeyer flasks. The flasks were incubated at 55 °C and 200 rpm for 5 days. The concentration of reducing sugars released was monitored throughout the hydrolysis process by the DNS method. At the end of the hydrolysis, the OP hydrolysates were centrifuged at 10 000 rpm for 30 min at 4 °C and the supernatant was collected and stored at 4 °C until its use for LA fermentation.

Lactic acid (LA) fermentation

Lactic acid bacteria (LAB) strains

Three LAB strains (*Lacticaseibacillus rhamnosus* 1019, *Lacticaseibacillus casei* 2246 and 2240, all isolated from Parmigiano Reggiano cheese) were used in mono- and co-culture for LA production from OP hydrolysates. All the strains used belong to the University of Parma Culture Collection (UPCC), an associated Member of the JUR MIRRI-IT. Microbial strains were maintained as frozen stocks (−80 °C) in Man Rogosa Sharpe (MRS) medium (Oxoid, Milan, Italy) supplemented with 12.5% glycerol (*w/v*). The cultures were propagated three times by using 3% (*v/v*) inoculum grown in MRS at 37 °C for 15 h. The starter inoculum for lacto-fermentation was prepared by cultivating the strains until the late exponential phase (*c.* 15 h), harvesting the cells by centrifugation (10 000 rpm for 10 min at 4 °C), washing them twice with Ringer's solution (Oxoid), and finally re-suspending the cells in sterile distilled water to the final concentration of 9.0 log CFU mL^{-1}.

Effect of yeast extract and calcium carbonate ($CaCO_3$) addition on lactic acid (LA) production

Lacticaseibacillus casei 2246 was employed for the preliminary tests since it demonstrated to be a good producer of LA in a previous study.[6] The cells obtained, as described earlier, were inoculated into the OP hydrolysates, previously sterilized in an autoclave (120 °C for 20 min), until an initial concentration of 7.0 log CFU mL^{-1}.

Two studies were conducted, performing all the fermentations in triplicate:

(1) Hydrolysate supplementation with yeast extract (nitrogen source). 10 g L^{-1} of yeast extract was added to the OP hydrolysate and the pH was adjusted to 6.5. The fermentation was conducted for 5 days at 37 °C and 150 rpm. LA concentration was measured each day for 5 days. The same experiment was also conducted without the addition of yeast extract.
(2) Calcium carbonate ($CaCO_3$) addition of hydrolysates for pH control. 0.5 or 1% *w/w* of $CaCO_3$ was added to the OP hydrolysate containing 10 g L^{-1} of yeast extract. The initial pH was adjusted to 6.5. Since in the previous experiment performed with yeast extract supplementation the highest concentration of LA was observed after 3 days, the fermentation was conducted for 3 days at 37 °C at 150 rpm. Microbial concentration, pH, reducing sugars and LA concentration were measured each day.

Productivity and yield were also calculated from the mean values of LA and reducing sugars concentrations as followed:

$$\text{Productivity}\left(\text{g L}^{-1}\text{h}^{-1}\right)=\frac{\text{lactic acid concentration}\left(\text{g L}^{-1}\right)}{\text{time (h)}},$$

$$\text{Yield}\,(\%)=\frac{\text{lactic acid concentration}\left(\text{g L}^{-1}\right)}{\textit{initial reducing sugars concentration}\left(\text{g L}^{-1}\right)}\times 100.$$

Effect of lactic acid bacteria (LAB) strains on lactic acid (LA) fermentation

Sterile OP hydrolysate was supplemented with 10 g L^{-1} yeast extract, with the pH adjusted to 6.5, inoculated with LAB strains to reach the initial concentration of 7.0 log CFU mL^{-1} and incubated at 37 °C and 150 rpm. Three different strategies of fermentation were performed to evaluate LA production and reducing sugars reduction (Fig. 1):

(1) Strategy 1. The fermentation was conducted employing mono-cultures of *L. rhamnosus* 1019, *L. casei* 2246 and 2240 for 3 days.
(2) Strategy 2. The fermentation was conducted employing mono-cultures of *L. rhamnosus* 1019, *L. casei* 2246 and 2240 for 2 days. Then the three strains were mixed in pairs (*L. rhamnosus* 1019/*L. casei* 2240, *L. rhamnosus* 1019/*L. casei* 2246 and *L. casei* 2240/*L. casei* 2246) and the fermentation was conducted for another day. For co-culture, equal volumes of each mono-cultures were mixed.
(3) Strategy 3. The fermentation was conducted employing mono-cultures of *L. rhamnosus* 1019 and *L. casei* 2240 for 2 days. At this time, recovered cells (8 log CFU mL^{-1}) of *L. casei* 2240 or *L. casei* 2246, collected from an overnight culture in a synthetic medium, were added and the fermentation was conducted for another day.

All the fermentations were performed in triplicate.

Microbial concentration, pH, reducing sugars and LA concentration were measured each day and each replicate was analysed three times. Productivity and yield were also calculated as reported earlier.

Lactic acid (LA) quantification

LA concentration during fermentation was determined by an anion-exchange chromatography system (930 Compact IC Flex; Metrohm, Herisau, Switzerland) coupled with a conductivity detector. The opportune dilutions were prepared using a solution of sulphuric acid (0.4 mmol L^{-1})/acetone (120 mL L^{-1}). The

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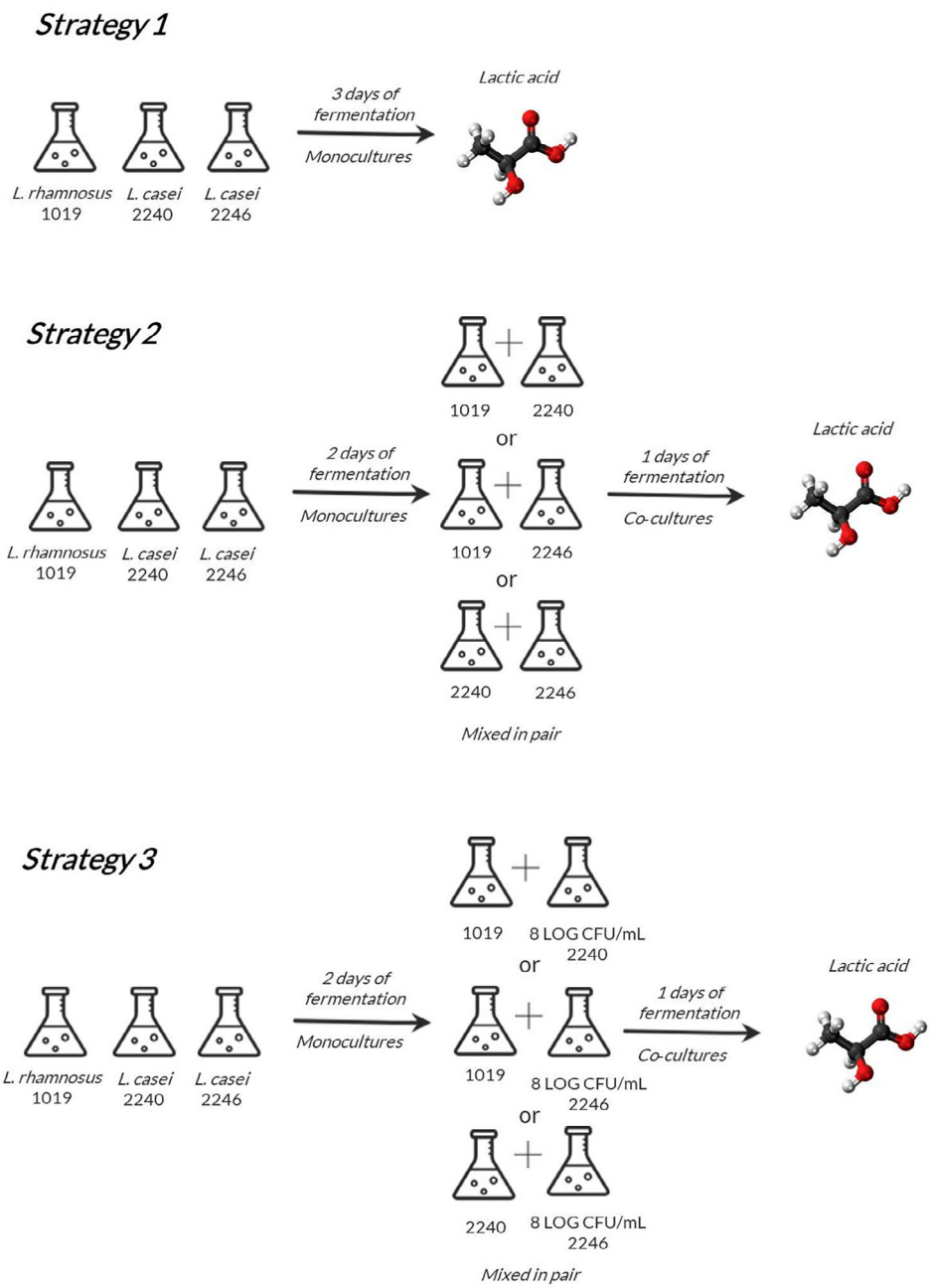

Figure 1. Experimental strategies for LA fermentations of OP hydrolysates.

separation of compounds was carried out in samples of 10 mL using a Metrosep Organic Acids 250/7.8 column (Metrohm). The eluent used was a solution of sulphuric acid (0.4 mmol L^{-1})/acetone (120 mL L^{-1}) with a flow rate of 0.4 mL min^{-1}. For the quantification, a calibration curve with an external standard (LA) was performed.

RESULTS AND DISCUSSION

Fibre analysis

Data reported in Table 1 highlight that OP is a by-product rich in pectins and proteins (89.97%), but it also contains a small proportion of cellulose (3.2%) and hemicellulose (3.39%), confirming the composition of OPs described in other published article.[6,29] The OP composition, rich in carbohydrates and with low lignin content, makes it a promising raw material for fermentable sugar production through enzyme hydrolysis. Lignin limits the access of enzymes to carbohydrates, contributing significantly to biomass recalcitrance.[30] After the washing step with tap water, the composition observed is different. Cellulose and soluble lignin fractions increased significantly, reaching 16.89%, while the hemicellulose fraction showed a slight increase (from 3.39% to 4.30%). These increments are due to the solubilization of the protein, pectin and salt fraction during washing. This fraction was reduced by more than 15%.

Enzyme activities

The enzymatic extracts obtained from fermented WOP contained a variety of hydrolytic enzymes secreted by *A. awamori*. The low

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concentration of simple sugars in WOPs and their high concentration of carbohydrate polymers, mainly cellulose, hemicellulose and pectin (Table 1), induce enzyme production by the fungus.[18] The enzymatic activities (IU g^{-1} of dried WOP) measured after 5 days of fermentation were: 40.6 ± 4.8 for xylanases, 16.3 ± 1.7 for exo-polygalacturonase (pectinase), 0.9 ± 0.5 for FPase and 0.58 ± 0.08 for *β*-glucosidase. Xylanase activity from fermented WOP was higher than the one measured for the same fungus but using other substrates. Thus, when *A. awamori* was grown on exhausted sugar beet cosettes, a xylanase activity of 35 IU g^{-1} was achieved, but 28 IU g^{-1} of exo-polygalacturonase was measured in this case.[31] Higher pectinase activities (657 IU g^{-1}), have been reported with *Hypocrea pseudokoningi* by using coffee husk.[32] As regards FP-ase, the activity measured in the present work is similar to that analysed for *Aspergillus tubingensis* JP-1, which was in the range 0.024–0.669 IU g^{-1}, grown for 10 days at 30 °C on wheat straw, wheat bran, rice bran and sugarcane bagasse.[33] Among the studied enzymes, *β*-glucosidase activity showed a lower value, although it was similar to the one measured for other residues. Therefore, activities below 0.018 IU g^{-1} were achieved with *A. niger* grown for 5 days at 30 °C on wheat bran, groundnut fodder, sawdust and rice bran.[34] Generally, enzymes produced in SSF are extracted and, in some cases, purified before being used in enzymatic hydrolysis. Given that enzyme extraction and purification count for the most cost of this process, in the present study, we explore the feasibility of direct use of fermented OPs for hydrolysis.

Enzymatic hydrolysis of orange peels (OPs)

Figure 2 shows the reducing sugar concentrations in the extracts with the hydrolysis time for the two fermented/non-fermented solid ratios assayed. A fast increase in reducing sugar was observed after the first day of hydrolysis and, from this moment, a slight increase was produced until the end of the process. Higher concentrations of reducing sugars were measured for 20% fermented WOP/80% OP, with a maximum value of 24.4 g L^{-1}, which was almost two times higher than for 50% fermented WOP/50% OP (14.8 g L^{-1}). These results could be due to the higher amount of potential hydrolysable polysaccharides available in the ratio of 20:80. In this case, there was a lower portion of fermented WOP, in which part of these polysaccharides were consumed by the fungus during SSF. Moreover, the non-fermented OP contained a higher concentration of monomeric sugars than WOP, which were partially removed during the washing step. In addition, despite the fungus growth being limited at the high temperature of hydrolysis (50°C), in the literature it is reported that some fungal species can grow at temperatures as high as 50 °C.[35,36] Thus, part of the monosaccharides released in the hydrolysis could have been consumed by the fungus, being this reduction more important at the highest proportion of fermented WOP. Similar results were observed in a previous study carried out by Marzo *et al.*[20] in which the maximum concentration of reducing sugars was lower when the fermented/unfermented ratio of sugar beet changed from 14.3%/85.7% to 50%/50%.

Table 1. Orange peels (OPs) and washed orange peels (WOPs) detergent fibre analysis

Fraction	OPs (%)	WOPs (%)
Fats, oil, etc.	3.44	2.72
Proteins, pectins, soluble salts, etc.	89.97	76.10
Soluble salts	3.57	5.21
Extractable material non-saline	86.40	70.90
Hemicellulose	3.39	4.30
Cellulose and soluble lignin	3.20	16.89
Insoluble lignin	n.d.	n.d.
Insoluble salts	n.d.	n.d.
Total salts	3.57	5.21
Total sum	100.00	100.00

Note: n.d., not detected.

Lactic acid (LA) fermentation

Effect of yeast extract on lactic acid (LA) production

The influence of OP hydrolysates supplementation with a nitrogen source (10 g L^{-1} of yeast extract) in LA production with *L. casei* 2246 was studied. The evolution of LA concentration over time is shown in Fig. 3. After 5 days of fermentation, the same concentration of LA was reached (*c.* 9.5 g L^{-1}) with and without supplementation. However, with the addition of yeast extract, the highest value was obtained just after 3 days of fermentation, being possible to reduce to 48 h the time needed to reach the maximum concentration. The presence of yeast extract increases the production rate of LA, which supposes productivity of 0.22 g L^{-1} h^{-1} and a yield of 50.2% after 3 days of fermentation. These results agree with those obtained in previous studies, in which supplementations with yeast extract increased the concentration of LA produced from different raw materials including unpolished rice, spent coffee, waste obtained during malting and brewing processing, etc.[4,37,38] The role of yeast extract seems not to be related to the additional amount of nitrogen source added, but to the increase of the vitamin content. Yeast extract can provide group B complex vitamins, and growth factors supporting LAB growth. Indeed, according to the literature, the more vitamins and growth factors are supplemented, the more biomasses and LA are produced.[39,40]

For the next experiments, 10 g L^{-1} of yeast extract was added to the hydrolysates and the fermentations were conducted for 3 days.

Effect of calcium carbonate ($CaCO_3$) addition during lactic acid (LA) production

It is well known that during LA fermentation there is a decline in pH as a result of LA production. This drop can cause the inhibition of microbial growth, therefore neutralizing agents, like $CaCO_3$, are added to the fermentation broth.[41,42] In the present work the influence of the addition of $CaCO_3$, at 0.5 and 1% *w/w*, in LA production using *L. casei* 2246 was investigated. The selection of this strain was based on a previous study in which *L. casei* 2246 demonstrated to be a good LA producer.[6] Figure 4 shows *L. casei* 2246 growth, the evolution of pH, the reducing sugars and LA concentration over the 3 days of fermentation. As can be seen, none of these parameters were strongly affected by $CaCO_3$ addition at the studied concentrations. Despite the growth of *L. casei* 2246, reducing sugars and LA concentrations were not influenced by the presence of $CaCO_3$, the pH decreased to a less extent. In fact, in all the conditions tested, the maximum LA concentration ranged between 12.7 ± 0.1 g L^{-1} (without $CaCO_3$) and 11.4 ± 0.2 g L^{-1} (with 1% of $CaCO_3$). Similarly, productivities and yields reflect the same trend (from 0.31 g L^{-1} h^{-1}, without $CaCO_3$, to 0.26 g L^{-1} h^{-1}, with 1% of $CaCO_3$, and from 54.9%, without $CaCO_3$, to 46.3%, with $CaCO_3$). This fact can be explained by the low pH of

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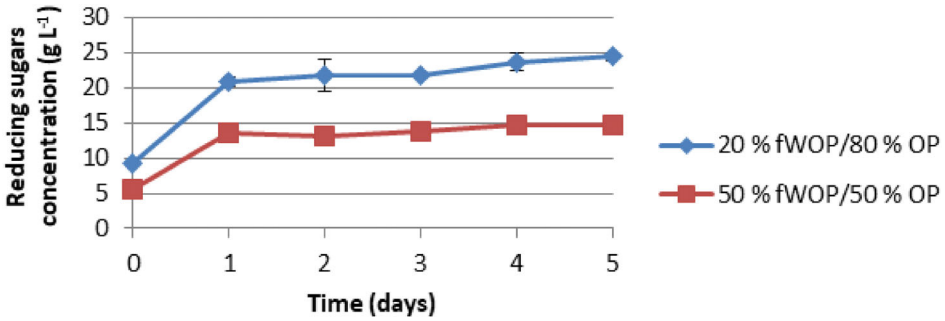

Figure 2. Evolution of reducing sugar concentration during enzymatic hydrolysis. The blue and red lines refer to the hydrolysis conducted with 20% of fermented WOP (fWOP) and 80% of OP and 50% of fWOP and 50% of OP, respectively.

the medium in all conditions, being below 5.0, which is outside the optimal range of LAB metabolism (pH 5.5–6.5).[43] Therefore, for example, LA production decreased at a pH value lower than 6.5.[6] Other authors have seen a positive effect of $CaCO_3$ at higher concentrations than those studied in the present article. John *et al.*[44] found that LA production was higher by increasing the percentage of $CaCO_3$ from 1% to 4%. Above this value, the production was kept constant. A positive effect has been observed by other authors at lower $CaCO_3$ concentrations. Therefore, for example, the final concentration of LA achieved from raw starch in wheat bran using *Lactobacillus amylophilus* GV6 was significantly improved when 0.375% $CaCO_3$ was added.[45] A decrease in LA production was observed at a higher concentration of $CaCO_3$, which was explained by the inhibition of the enzymes involved in LA biosynthesis. Some authors have reported that a high concentration of $CaCO_3$ affects the growth of microorganisms.[46] Sridevi *et al.*[47] observed a positive effect on LA production from cassava flour by *Lactobacillus plantarum* JX183220 by increasing $CaCO_3$ concentration from 0.1% to 0.3%. However, it decreased from 0.3% to 0.5%.

Given the results obtained, the following fermentations were performed by adding 10 g L^{-1} of yeast extract to the OP hydrolysate and adjusting the initial pH of hydrolysates to 6.5 without the addition of $CaCO_3$.

Effect of lactic acid bacteria (LAB) strain used in mono- or co-culture during lactic acid (LA) production

Different LAB strains (*L. casei* 2246, 2240 and *L. rhamnosus* 1019) in mono-culture (strategy 1) and co-culture (strategies 2 and 3) were tested for LA production (Fig. 1). In strategy 2, two cultures of two different strains were cultivated in mono-culture for 2 days and then cultivated in co-culture for another day, while, in strategy 3, the fresh cells of a strain were added to a 2-days culture of another strain and cultivated in co-culture for another day.

All strains demonstrated a good growth ability in the hydrolysates, reaching the maximum cell concentration after 1 day of fermentation (*c.* 9 log CFU mL^{-1}) and remaining practically constant for the next days (Table 2). Also, the highest decrease in pH was recorded in the first 24 h, decreasing more slowly for the rest 2 days but maintaining below pH 5 (Table 2).

Considering the mono-cultures, *L. casei* strains produced higher LA concentrations in less time of fermentation than *L. rhamnosus* 1019 (Table 3). LA production increased for the first 2 days for *L. casei* strains, but later a slight reduction was observed. However, in the case of *L. rhamnosus* 1019, LA production increased continuously during the 3 days of culture. On the second day of fermentation, LA concentrations achieved values above 10 g L^{-1} for both *L. casei* strains, attaining the highest one with *L. casei* 2466 (10.72 g L^{-1}). In this case, a 74.1% consumption of reducing sugar was achieved, which was 32.2% and 6.43% higher, than the ones for *L. rhamnosus* 1019 and *L. casei* 2240, respectively. Also, the highest productivity and yield were calculated for the same strain (0.26 g L^{-1} h^{-1} on day 1, and 40% on day 2, respectively) (Table 4).

Regarding the co-cultures, *L. casei* 2240 and, especially, *L. rhamnosus* 1019 provided better results in co-culture. For both strains, the co-culture with *L. casei* 2246 allowed to obtain LA concentrations of 10.52 (with 72.7% reducing sugar consumption) and 10.05 g L^{-1} (61.2% reducing sugar consumption), respectively, using strategy 2. With this strategy, the maximum LA concentration (10.59 g L^{-1}) was measured for the co-culture of *L. rhamnosus* 1019 with *L. casei* 2240.

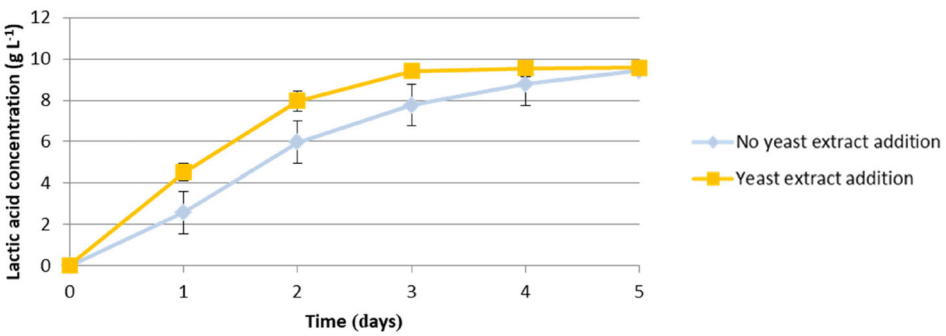

Figure 3. Effect of yeast extract supplementation in LA production. The orange and blue lines refer to experiments conducted with yeast extract and without supplementation, respectively.

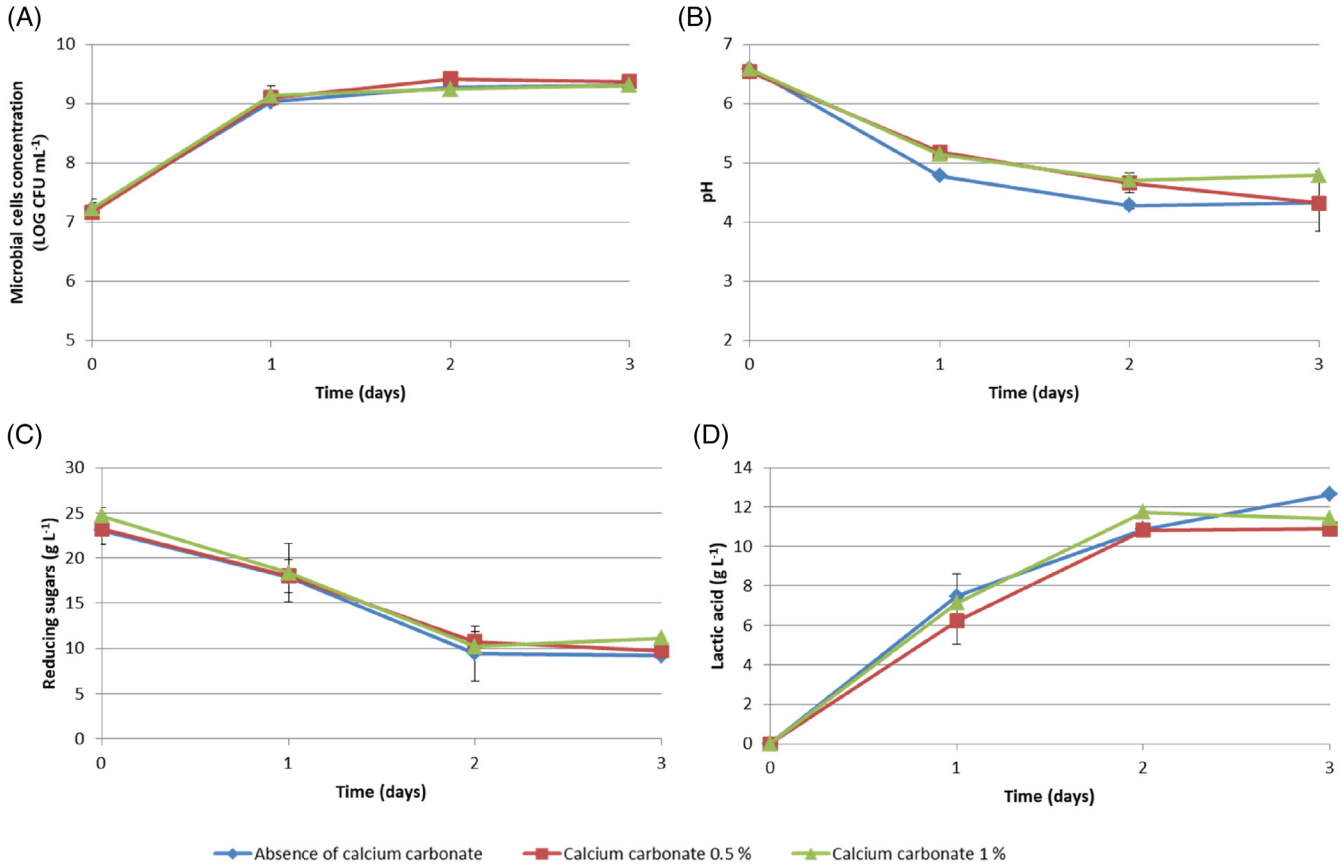

Figure 4. Effect of $CaCO_3$ addition to OP hydrolysates supplemented with yeast extract (10 g L^{-1}) at an initial pH of 6.5 (0% blue line, 0.5% red line and 1% green line). *Lacticaseibacillus casei* 2246 growth (A), pH (B), reducing sugars concentration (C) and LA concentration (D).

Strategy 3 provided slightly lower LA concentration than strategy 2 for the co-culture of *L. casei* 2240 and *L. casei* 2246 (from 10.52 to 10.28 g L^{-1}) and *L. casei* 2240 co-cultivated with *L. rhamnosus* 1019 (from 10.59 to 10.25 g L^{-1}). However, the co-culture of *L. casei* 2246 with *L. rhamnosus* 1019 with strategy 3 allowed to obtain a higher concentration of LA than with strategy 2 (10.25 *versus* 10.05 g L^{-1}).

The co-culture approach has been investigated by other authors. As scientific literature suggests, the employment of mixed cultures can improve LA production, due to more efficient

Table 2. Microbial concentration (log CFU mL^{-1}) and pH during lactic acid (LA) fermentation of orange peel (OP) hydrolysate with *Lacticaseibacillus casei* 2246, *L. casei* 2240 and *L. rhamnosus* 1019 in mono- and co-culture

		Day 0	Day 1		Day 2		Day 3	
Strategy	Strains	CFU mL^{-1}	CFU mL^{-1}	pH	CFU mL^{-1}	pH	CFU mL^{-1}	pH
1 (mono-culture)	2246	7.44 ± 0.09	9.26 ± 0.05	4.69 ± 0.10	9.36 ± 0.08	4.38 ± 0.04	9.21 ± 0.14	4.39 ± 0.12
	2240	7.57 ± 0.05	9.33 ± 0.05	4.74 ± 0.05	9.39 ± 0.09	4.12 ± 0.12	9.13 ± 0.11	4.36 ± 0.01
	1019	7.45 ± 0.09	9.14 ± 0.04	4.84 ± 0.04	9.09 ± 0.06	4.11 ± 0.07	9.24 ± 0.30	4.07 ± 0.01
2 (co-culture)	2246/	7.47 ± 0.08	9.30 ± 0.09	4.65 ± 0.10	9.27 ± 0.02	4.38 ± 0.03	9.39 ± 0.06	3.94 ± 0.02
	2240	7.55 ± 0.15	9.38 ± 0.08	4.78 ± 0.07	9.35 ± 0.05	4.12 ± 0.07		
	2246/	7.48 ± 0.05	9.16 ± 0.06	4.65 ± 0.10	9.25 ± 0.07	4.25 ± 0.19	9.18 ± 0.04	3.93 ± 0.02
	1019	7.55 ± 0.13	9.18 ± 0.04	4.88 ± 0.04	9.06 ± 0.07	4.18 ± 0.04		
	2240/	7.65 ± 0.05	9.34 ± 0.05	4.73 ± 0.05	9.27 ± 0.08	4.12 ± 0.01	9.19 ± 0.04	3.91 ± 0.02
	1019	7.50 ± 0.10	9.24 ± 0.04	4.85 ± 0.08	9.10 ± 0.09	4.15 ± 0.03		
3 (co-culture)	2240/	7.49 ± 0.06	9.40 ± 0.12	4.45 ± 0.03	9.47 ± 0.07	4.10 ± 0.02	9.26 ± 0.04	3.97 ± 0.04
	2246	—	addition of *L. casei* 2246 after 2 days of fermentation (8 log CFU mL^{-1})					
	1019/	7.60 ± 0.08	9.09 ± 0.09	4.99 ± 0.09	9.09 ± 0.15	4.19 ± 0.04	9.05 ± 0.07	3.98 ± 0.01
	2246	—	addition of *L. casei* 2246 after 2 days of fermentation (8 log CFU mL^{-1})					
	1019—	7.44 ± 0.09	9.09 ± 0.06	4.93 ± 0.07	9.16 ± 0.07	4.24 ± 0.08	9.03 ± 0.07	
	2240	—	addition of *L. casei* 2240 after 2 days of fermentation (8 log CFU mL^{-1})					

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Table 3. Lactic acid (LA) and reducing sugar (RS) concentration (g L^{-1}) during LA fermentation of orange peel (OP) hydrolysate with *Lacticaseibacillus casei* 2246, *L. casei* 2240 and *L. rhamnosus* 1019 in mono- and co-cultures

		Day 1			Day 2			Day 3		
Strategy	Strains	RS	RS decrease (%)	LA	RS	RS decrease (%)	LA	RS	RS decrease (%)	LA
1 (mono-culture)	2246	14.80 ± 1.07	44.3	6.24 ± 0.13	6.88 ± 0.28	74.1	10.72 ± 0.46	6.93 ± 0.41	73.9	9.86 ± 0.13
	2240	14.45 ± 0.55	45.7	5.61 ± 0.16	7.52 ± 0.32	71.7	10.03 ± 0.43	7.70 ± 0.14	71.0	9.20 ± 0.29
	1019	21.10 ± 0.79	20.7	4.06 ± 0.19	13.15 ± 0.96	50.6	7.27 ± 0.20	11.79 ± 1.46	55.7	9.20 ± 0.29
2 (co-culture)	2246/	14.81 ± 0.87	44.3	6.25 ± 0.11	6.89 ± 0.22	74.1	10.62 ± 0.36	7.26 ± 0.39	72.7	10.52 ± 0.42
	2240	14.35 ± 0.55	45.7	5.65 ± 0.16	7.45 ± 0.12	71.7	10.12 ± 0.45			
	2246/	14.50 ± 1.00	44.3	6.14 ± 0.13	6.87 ± 0.28	74.1	10.61 ± 0.48	10.32 ± 1.64	61.2	10.05 ± 0.08
	1019	20.90 ± 0.79	20.7	4.07 ± 0.29	13.05 ± 0.87	50.6	7.29 ± 0.20			
	2240/	14.12 ± 0.65	45.7	5.57 ± 0.14	7.42 ± 0.32	71.7	10.11 ± 0.43	8.78 ± 0.30	67.0	10.59 ± 0.47
	1019	21.18 ± 0.23	20.7	4.16 ± 0.17	12.99 ± 0.86	50.6	7.25 ± 0.27			
3 (co-culture)	2240/	15.32 ± 0.49	42.4	5.70 ± 0.16	9.87 ± 0.58	62.9	9.40 ± 0.42	7.84 ± 0.84	70.5	10.28 ± 0.12
	2246	addition of *L. casei* 2246 (8 log CFU mL^{-1}) after 2 days of fermentation with *L. casei* 2240								
	1019/	18.21 ± 0.72	31.6	4.00 ± 0.02	12.80 ± 0.94	51.9	7.65 ± 0.05	10.60 ± 1.78	60.2	10.25 ± 0.74
	2246	addition of *L. casei* 2246 (8 log CFU mL^{-1}) after 2 days of fermentation with *L. rhamnosus* 1019								
	1019/	19.05 ± 1.61	28.4	3.88 ± 0.14	12.71 ± 1.12	52.2	7.82 ± 0.33	9.05 ± 1.06	66.0	10.25 ± 0.74
	2240	addition of *L. casei* 2240 (8 log CFU mL^{-1}) after 2 days of fermentation with *L. rhamnosus* 1019								

Note: RS decrease, reducing sugars decrease during fermentation.

Table 4. Productivity and yield during fermentation with mono- and co-culture

Strains	Day 1		Day 2		Day 3	
	Productivity (g L^{-1} h^{-1})	Yield (%)	Productivity (g L^{-1} h^{-1})	Yield (%)	Productivity (g L^{-1} h^{-1})	Yield (%)
2246	0.260 ± 0.006	23.458 ± 0.566	0.223 ± 0.011	40.297 ± 1.941	0.137 ± 0.002	37.061 ± 0.538
2240	0.234 ± 0.008	21.105 ± 0.694	0.209 ± 0.010	37.721 ± 1.811	0.128 ± 0.003	34.580 ± 0.894
1019	0.169 ± 0.009	15.267 ± 0.793	0.152 ± 0.005	27.347 ± 0.859	0.128 ± 0.005	34.580 ± 1.231
2246/2240	—	—	—	—	0.146 ± 0.007	39.555 ± 1.773
2240/1019	—	—	—	—	0.147 ± 0.007	39.807 ± 1.996
2246/1019	—	—	—	—	0.140 ± 0.001	37.795 ± 0.320
1019/2240	addition of *L. casei* 2240 after 2 days of fermentation (8 log CFU mL^{-1})				0.142 ± 0.012	38.523 ± 3.131
1019/2246	addition of *L. casei* 2246 after 2 days of fermentation (8 log CFU mL^{-1})				0.142 ± 0.012	38.523 ± 3.131
2240/2246	addition of *L. casei* 2246 after 2 days of fermentation (8 log CFU mL^{-1})				0.143 ± 0.002	38.631 ± 0.513

Note: The results were expressed as the mean value ± the confidence limit for a probability of 95%.

sugar consumption, increasing the conversion efficiency of the substrates.[48] This effect has been observed in the present study mainly for *L. rhamnosus* 1019, which showed a sugar consumption in mono-culture of 55.7% and increased to 61.2–70.5% in co-culture (Table 3). In this case, the LA yield increased significantly from mono-culture (34.58%) to co-culture with *L. casei* 2240 or *L. casei* 2246 in both strategies assayed. Specifically, in the co-culture with *L. casei* 2240 by strategy 2 the yield increases by 15%.

Nevertheless, the highest concentration of LA was reached with *L. casei* 2246 in mono-culture. The main advantages of working in mono-culture are that it is possible to evaluate the maximum biomass and LA production of a specific strain. Also, it is easier to prepare the inoculum, follow the evolution of the fermentation and detect and control contaminations. One of the main disadvantages of co-culture is the control of the optimum balance among the microorganisms involved.

Recently, different studies have been published regarding the use of OP hydrolysates for LA production.[39,49,50] Generally, higher LA concentrations, as well as productivity and yield, were obtained than in the present study. Therefore, for example, *Lactobacillus delbrueckii* ssp. *delbrueckii* CECT 286 and *L. delbrueckii* ssp. *bulgaricus* CECT 5037 produced 45 and 39 g L^{-1} of LA in OP hydrolysates, respectively.[49] However, these strains were grown in OP hydrolysates supplemented with MRS (15% *v/v*) and 5 g L^{-1} meat extract. Also, around 45 g L^{-1} of LA was achieved with resting cells of *L. delbrueckii* spp. *delbrueckii* grown in OP hydrolysates in a bioreactor,[51] where the pH was maintained at 5.8 during the whole process, which could have favoured LA production.

In the present work, the hydrolysate was not supplemented with complex media, like MRS or meat extract, which possesses a very high concentration of reducing sugars (almost four times higher, in some cases than in our study) which are used for bacterial growth and LA production. Also, in our study the pH was not controlled along the fermentation, being below 4.0 in many of the conditions tested (Table 2), which is outside the optimal range for LA production (pH 5.5–6.5).[43]

In most published articles that use OP hydrolysates for fermentations, the sugars are generated by using different commercial enzymes cocktails, in most cases Celluclast 1.5 l, Novozym 188 or Pectinex Ultra SP-L, which generally contain cellulases, *β*-glucosidase, xylanase, *β*-xylosidase, pectinase and auxiliary activities.[49] The cost of these commercial cocktails is relevant, in fact, it is well-known that the current high cost of enzymes used in lignocellulose conversion is the main bottleneck in the biomass bioconversion to high-added value products.[52] As an alternative, in the present work, a cost-effective process is proposed as the same enzymes produced by fungal SSF on the OP were used to hydrolyse more OP and produce fermentable sugars. Moreover, with the direct addition of fermented OP as a source of enzymes, the stages of enzyme extraction and purification are not required. Despite commercial enzymes being more effective in the release of reducing sugars, with the optimization of the steps involved in the approach proposed in this study LA production could be improved by using a more cost-effective process.

CONCLUSION

To the best of our knowledge, this is the first study exploiting OPs as low-cost raw material for the production of LA avoiding the employment of commercial enzymes. For this purpose, OPs were fermented with *A. awamori* in the solid state and directly used for the hydrolysation of non-fermented OPs. Indeed, during fermentation, the fungus produced different enzymes, mainly xylanase and exo-polygalacturonase, exploited for the release of fermentable sugars during the hydrolytic step from OPs. The hydrolysate obtained was lacto-fermented by three strains of LAB, in mono- and co-cultures, and the highest concentration of LA was achieved with *L. casei* mono-culture. Nonetheless, this is a preliminary work carried out to study the feasibility of this approach while in future studies the optimization of the hydrolyses, as well as the condition to obtain the highest LA concentrations will be studied.

AUTHOR CONTRIBUTIONS

Annalisa Ricci did all the experimental work. The experimental design was performed by Ana Belen Diaz and Ana Blandino. The analysis and interpretation of the results was performed by Ana Blandino, Annalisa Ricci and Camilla Lazzi. The manuscript was written by Annalisa Ricci, Ana Blandino and Ana Belen Diaz and the revision of the manuscripts was carried out by Camilla Lazzi, Ana Blandino and Ana Belen Diaz.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The results/data/figures in this manuscript have not been published elsewhere.

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