**Characterization of a versatile arylesterase from *Lactobacillus*
plantarum active on wine esters**

MARÍA ESTEBAN-TORRES[†], JOSÉ MARÍA BARCENILLA[†], JOSÉ MIGUEL
MANCHEÑO[‡], BLANCA DE LAS RIVAS[†], ROSARIO MUÑOZ^{†,*}

[†] Laboratorio de Biotecnología Bacteriana, Instituto de Ciencia y Tecnología de Alimentos
y Nutrición, ICTAN-CSIC, Juan de la Cierva 3, 28006 Madrid, Spain

[‡] Grupo de Cristalografía y Biología Estructural, Instituto de Química-Física
“Rocasolano”, IQFR-CSIC, Madrid, Spain

* Corresponding author:

Tel: +34 91 5622900; Fax: +34 91 564 4853.

E-mail: rmunoz@ifi.csic.es

Abstract

The gene *lp_1002* from *Lactobacillus plantarum* WCFS1 encoding a putative lipase/esterase was cloned and overexpressed in *Escherichia coli* BL21(DE3). The purified Lp_1002 protein was biochemically characterized. Lp_1002 is an arylesterase which showed high hydrolytic activity on phenyl acetate. Although to a lesser extent, Lp_1002 also hydrolyzed most of the esters assayed including relevant wine aroma compounds. Importantly, Lp_1002 exhibited hydrolytic activity at winemaking conditions, although optimal catalytic activity is observed at 40 °C and pH 5-7. The effect of wine compounds on Lp_1002 activity was assayed. From the compounds assayed (ethanol, sodium metabisulfite, and malic, tartaric, lactic and citric acids), only malic acid slightly inhibited Lp_1002 activity. Lp_1002 is the first arylesterase described in a wine lactic acid bacteria and possessed suitable biochemical properties to be used during winemaking.

KEYWORDS: Ethyl acetate, Esterase, Flavour, Lactic acid bacteria, Wine aroma

INTRODUCTION

Esters constitute a large group of compounds that are usually present in wine at concentrations above the sensory threshold. Wine esters are derived from the grape, from the chemical esterification of alcohols and acids during wine aging,¹ and from the yeast and bacterial metabolism during vinification.² The importance of esters in winemaking lies in their prominent role in determining the aroma and, by extension, the quality of wine. Esters are responsible for the desirable, fruity aroma of young wines, although they can also have a detrimental effect on wine aroma when they are present at excessive concentration.³

Ester hydrolysis and synthesis can be catalysed by carboxylesterases.⁴ Carboxylic ester hydrolases (EC 3.1.1.x) comprise a valuable source of enzymes which mainly belong to the α/β hydrolase fold superfamily of enzymes, which use water to hydrolyze ester bonds in aqueous solutions forming an alcohol and a carboxylic acid. Examples of carboxylic ester hydrolases are carboxylesterases (EC 3.1.1.1), arylesterases (EC 3.1.1.2) and lipases (EC 3.1.1.3).⁵ Carboxylesterases and arylesterases typically catalyse the hydrolysis of water-soluble and short- to medium-length aliphatic esters, and can be distinguished by the ability of the latter enzymes to preferentially hydrolyze aromatic esters. In contrast, lipases typically display high activity towards water-insoluble long-chain esters.⁶

While the esterases from yeasts have been extensively studied,⁷ esterase activity for wine-related lactic acid bacteria (LAB) is not well documented. Most characterization of esterases from LAB has focused on dairy isolates.⁸⁻¹¹ In winemaking, the malolactic fermentation used to deacidify wine is typically carried out by *Lactobacillus* spp., *Pediococcus* spp., and particularly *Oenococcus oeni* strains.¹² Esterases from wine

Oenococcus oeni and *Lactobacillus hilgardii* strains have been characterized.¹³⁻¹⁴ Among wine LAB, besides *O. oeni*, *Lactobacillus plantarum* strains are also used sometimes as malolactic starters. *L. plantarum* is a good source of esterase enzymes; in fact, lipases,¹⁵⁻²¹ esterases,²²⁻²³ acetyl esterases,²⁴ carboxylesterase,²⁵ or feruloyl esterase²⁶ proteins have been purified and characterized.

Despite numerous genome sequences from *L. plantarum* are currently available, there is still limited information on the function of genes coding for esterases and their potential contribution to food and beverage aroma. Many of the putative gene products have been annotated based on sequence comparisons with other proteins functionally characterized. Although this approach is fast and inexpensive, over 40% of sequences typically fail to be assigned a function, and even many open reading frames are incorrectly annotated.²⁷ Moreover, since nowadays prediction of the specific biochemical features of the coded proteins based exclusively on their amino acid sequences or even their three dimensional structures is not feasible, the definitive approach to assigning a specific molecular function to a predicted open reading frame is to biochemically characterize the corresponding protein. In this regard, the objective of this study was to determine the functional features of the putative esterase/lipase Lp_1002 from *L. plantarum* WCFS1, through biochemical characterization of the recombinantly expressed protein. With a view to applying this esterase under conditions found in wine, enzyme activity under physicochemical conditions frequently encountered in wine was studied.

MATERIALS AND METHODS

Bacterial strains, plasmids, enzymes, and reagents. *L. plantarum* WCFS1 was kindly provided by M. Kleerebezem (NIZO Food Research, The Netherlands).

Escherichia coli DH10B and *E. coli* BL21 (DE3) were used as transformation and expression hosts in the pURI3-Cter vector.²⁸ Plasmid pGro7 (TaKaRa) was used to overexpress GroES/GroEL chaperones. The *L. plantarum* strain was grown in MRS medium (Pronadisa, Spain) adjusted to pH 6.5 and incubated at 30 °C. The *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37 °C and shaking at 200 rpm. When required, ampicillin, chloramphenicol, or arabinose were added to the medium at a concentration of 100, 20 µg/mL, or 2 mg/mL, respectively.

Plasmid DNA was extracted by a High Pure plasmid isolation kit (Roche). PCR product was purified with a QIAquick gel extraction kit (Qiagen). Oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany). *DpnI* and HS Prime Star DNA polymerase were obtained from TaKaRa. His-tagged protein was purified by a Talon Superflow resin (Clontech). All the *p*-nitrophenyl esters, isopropyl-β-D-thiogalactopyranoside (IPTG), ampicillin, chloramphenicol, and arabinose were obtained from Sigma (Madrid, Spain). All other chemicals were obtained from commercial suppliers.

Cloning of *lp_1002* from *L. plantarum*. The gene encoding for a putative lipase/esterase (*lp_1002*) (gene ID 1063602) in *L. plantarum* WCFS1 was PCR-amplified by Prime Star HS DNA polymerase (TaKaRa) by using the primers 959 (5'-*TAACTTTAAGAAGGAGATATACATatgcaagttattaagcaaaaattaa*) and 960 (5'-*GCTATTAATGATGATGATGATGATGacgattatcagctagccattcaag*) (the nucleotides pairing the expression vector sequence are indicated in italics, and the nucleotides pairing the *lp_1002* gene sequence are written in lowercase letters). The 795-bp purified PCR product was inserted into the pURI3-Cter vector using a restriction enzyme- and ligation-free cloning strategy.²⁸ The vector produce recombinant proteins having a six-histidine affinity

116 tag in their C-termini. *E. coli* DH10B cells were transformed, recombinant plasmids were
117 isolated, and those containing the correct insert were identified by restriction enzyme
118 analysis, verified by DNA sequencing, and then transformed into *E. coli* BL21 (DE3) cells
119 for expression.

120

121 **Expression and purification of recombinant Lp_1002 esterase.** *E. coli*

122 BL21(DE3) harbouring pGro7 (TaKaRa), a vector overexpressing GroES/GroEL
123 chaperones, was transformed with the recombinant plasmid pURI3-Cter-1002. *E. coli* was
124 grown in LB medium containing 100 µg/mL ampicillin, 20 µg/mL chloramphenicol, and 2
125 mg/mL arabinose until an optical density at 600 nm of 0.4 was reached and then induced
126 by adding isopropyl-β-D-thiogalactopyranoside (IPTG) at 0.4 mM final concentration.
127 Following induction, the cells were grown at 22 °C for 20 h and collected by
128 centrifugation (8,000 g, 15 min, 4 °C). The cells were resuspended in phosphate buffer (50
129 mM, pH 7) containing 300 mM NaCl. Crude extracts were prepared by French press lysis
130 of the cell suspension (three times at 1,100 psi). The insoluble fraction of the lysate was
131 removed by centrifugation at 47,000 g for 30 min at 4 °C, and the supernatant was filtered
132 through a 0.2 µm pore-size filter and then applied to a Talon Superflow resin (Clontech)
133 equilibrated in phosphate buffer (50 mM, pH 7) containing 300 mM NaCl and 10 mM
134 imidazole to improve the interaction specificity in the affinity chromatography step. The
135 bound enzyme was eluted using 150 mM imidazole in the same buffer. The purity of the
136 enzymes was determined by SDS-PAGE in Tris-glycine buffer. Protein concentration was
137 measured according to the method of Bradford using a protein assay kit (Bio-Rad) with
138 bovine serum albumin as the standard. Fractions containing the His6-tagged protein were
139 pooled and analyzed for esterase activity.

140

141 **Enzyme assay.** Esterase activity was determined by a spectrophotometric method
142 described previously using *p*-nitrophenyl acetate (Sigma-Aldrich) as the substrate.²⁶ The
143 rate of hydrolysis of *p*-nitrophenyl acetate for 10 min at 37 °C was measured in 50 mM
144 sodium phosphate buffer pH 7.0 at 348 nm in a spectrophotometer (UVmini-1240
145 Shimadzu). The reaction was stopped by chilling on ice.

146 In order to carry out the reaction (1 mL), a stock solution of 25 mM of *p*-
147 nitrophenyl acetate was prepared in acetonitrile/isopropanol (1:4, v/v)²⁹ and mixed with 50
148 mM sodium phosphate buffer (pH 7.0) to obtain a 1 mM substrate final concentration.
149 Control reactions containing no enzyme were utilized to account for any spontaneous
150 hydrolysis of the substrates tested. Enzyme assays were performed in triplicate. One unit
151 of esterase activity was defined as the amount of enzyme required to release 1 μmol of *p*-
152 nitrophenol per minute under standard reaction conditions

153
154 **Substrate specificity.** The substrate specificity of Lp_1002 was determined by
155 using different *p*-nitrophenyl esters of various chain lengths (Sigma-Aldrich): *p*-
156 nitrophenyl acetate (C2), *p*-nitrophenyl butyrate (C4), *p*-nitrophenyl caprylate (C8), *p*-
157 nitrophenyl laurate (C12), *p*-nitrophenyl myristate (C14) and *p*-nitrophenyl palmitate
158 (C16) as substrates as described previously.^{22,26} A stock solution of each *p*-nitrophenyl
159 ester was prepared in acetonitrile/isopropanol (1/4, v/v). Substrates were emulsified to a
160 final concentration of 0.5 mM in 50 mM sodium phosphate buffer, pH 7.0, containing 1.1
161 mg/mL Arabic gum, and 4.4 mg/mL Triton X-100. The reaction mix consisted of 990 μL
162 of emulsified substrate and 10 μL of enzyme solution (10 μg protein). Reactions were
163 carried out at 37 °C in a spectrophotometer (UVmini-1240 Shimadzu) as described above.

164 The enzymatic substrate profile of purified protein was determined by using an
165 ester library described previously.³⁰ *p*-Nitrophenol was used as pH indicator to monitor

166 ester hydrolysis colorimetrically. The screening was performed in a 96-well Flat Bottom
167 plate (Sarstedt) where each well contains a different substrate (1 mM) in acetonitrile (1%).
168 A buffer/indicator solution containing 0.44 mM of *p*-nitrophenol in 1 mM sodium
169 phosphate buffer pH 7.2 was used as pH indicator. Esterase solution 10 μ g (20 μ L in 1
170 mM sodium phosphate buffer pH 7.2) was added to each well and reactions were followed
171 by measuring the decrease in absorbance at 410 nm for 2 h at 37 °C in a Synergy HT
172 BioTek microplate spectrophotometer. Blanks without enzyme were carried out for each
173 substrate and data were collected in triplicate and the average activities were quantified.
174 Results are shown as means $\pm$ standard deviations.

175

176 **Effects of temperature, pH, and additives on Lp_1002 esterase activity.** The
177 effects of pH and temperature on the esterase activity of Lp_1002 were studied by using
178 buffers of different pH ranging from 3 to 9. The buffers (100 mM) used were acetic acid-
179 sodium acetate (pH 3-5), sodium phosphate (pH 6 to 7), Tris-HCl (pH 8), and glycine-
180 NaOH (pH 9). The optimal temperature was assayed by incubating purified Lp_1002
181 esterase in 50 mM phosphate buffer (pH 7) at different temperatures (5, 20, 30, 37, 40, 45,
182 55 and 65 °C). For temperature stability measurements, the recombinant esterase was
183 incubated in 50 mM phosphate buffer pH 7 at 20, 30, 37, 45, 55 and 65 °C for 5, 15, and
184 30 min and 1, 2, 4, 6, and 20 h. Aliquots were withdrawn at these incubation times to test
185 the remaining activity at standard conditions. The non-heated enzyme was considered as
186 control (100%). The analyses were performed in triplicate.

187 To study the effect of metals and ions on Lp_1002 esterase activity, the enzyme
188 was incubated in the presence of the different additives at a final concentration of 1 mM
189 for 5 min at room temperature. Then, the substrate was added, and the reaction mixture
190 was incubated at 37 °C. The residual esterase activity was measured after the incubation of

the purified enzyme with each additive. The additives analyzed were MgCl₂, KCl, CaCl₂, HgCl₂, ZnCl₂, CuCl₂, NiCl₂, MnCl₂, Triton-X-100, Tween 20, Tween 80, SDS, urea, EDTA, DMSO, cysteine, DTT, PMSF, DEPC, and β-mercaptoethanol. Esterase activity measured in the absence of any additive was taken as control (100%). Experiments were done in triplicate.

196

Effect of wine compounds on Lp_1002 activity. The effect of the presence of compounds present during winemaking was assayed. The effect of ethanol on esterase activity was studied at ethanol concentrations ranging from 0 to 20% (vol/vol). Reaction mixtures were prepared as described for the temperature optimum experiments, but different volumes of ethanol were added, and the volume of the buffer was adjusted accordingly to maintain the final reaction volume. The reactions were pre-incubated 5 min at room temperature, and the substrate (*p*-nitrophenyl acetate) was added. The effect of the presence of several organic acids was also studied. L-Malic, L-tartaric, DL-citric, and DL-lactic acids were assayed at concentrations ranging from 0 to 5 g/L. Reaction mixtures were prepared by adding different volumes of the corresponding stock solution (25 g/L). Finally, the effect of sulfite was determined by adding sodium metabisulfite at concentrations ranging from 0 to 1 g/L. The reactions were done by adding different volumes of a 25 g/L stock solution. Reactions mixtures were pre-incubated for 5 min at room temperature before the enzyme was added. After the reaction, the absorbance was measured.

212

Statistical analyses. The two-tailed Student's *t* test preformed using GraphPad InStat version 3.0 (GraphPad Software, San Diego, CA), was used to determine the differences

215 between means. The data are representative means of at least three independent experiments.

216

217 RESULTS AND DISCUSSION

218

219 **Production and purification of recombinant Lp_1002 protein.** Numerous open
220 reading frames (ORF) encoding putative lipase/esterase proteins can be identified from the
221 genomic information of complete *L. plantarum* genome sequences. Considering *L.*
222 *plantarum* WCFS1 genome (accession NC_004567), more than 20 ORF were annotated as
223 putative esterases. The first two ORFs lipase/esterase have been already biochemically
224 characterized, the feruloyl esterase Lp_0796^{13,26} and the esterase Lp_0973.²² The next
225 ORF annotated as putative esterase/lipase is Lp_1002, present in all the available *L.*
226 *plantarum* genome sequences, and predicted to encode a 260 amino acid sequence protein
227 with a theoretical molecular mass of 28.7 kDa. The deduced amino acid sequence of
228 Lp_1002 lacked a N-terminal secretion signal sequence suggesting that this enzyme is
229 located intracellularly. The amino acid sequence of Lp_1002 showed a 53% identity to
230 *Lactobacillus brevis* and *Lactobacillus coryniformis* proteins, which are annotated as
231 putative lipases/esterases (data not shown). The presence of the sequence conserved motif
232 Gly-X-Ser-X-Gly, typical of serine hydrolases, suggests they belong to the α/β hydrolase
233 superfamily of enzymes, what has been confirmed structurally since the atomic
234 coordinates of Lp_1002 have been deposited at the Protein Data Bank by the Joint Center
235 for Structural Genomics (PDB entry: 3bjr).

236 The *lp_1002* gene was cloned into the pURI3-Cter expression vector by a ligation
237 –free cloning strategy described previously.²⁸ The vector incorporates the DNA sequence
238 encoding a C-terminal hexa-histidine tail to create His-tagged fusion enzyme for further
239 purification step. The integrity of the construct was confirmed by DNA sequencing. The

240 *lp_1002* gene was expressed in *E. coli* under the control of an IPTG inducible promoter.
241 Cell extracts were used to detect the presence of overproduced proteins by SDS-PAGE
242 analysis. Whereas control cells containing the pURI3-Cter vector did not show protein
243 overexpression, an overproduced protein with an apparent molecular mass around 28 kDa
244 was present as inclusion bodies in the insoluble fraction. In order to produce Lp_1002 in a
245 soluble form, plasmid pGro7, producing GroES/GroEL chaperones, was used. When
246 pURI3-Cter-1002 and pGro7 plasmids were used simultaneously, Lp_1002 appeared in
247 the intracellular soluble fraction of the cells (**Figure 1**). Since the cloning strategy would
248 yield a His-tagged protein, *L. plantarum* Lp_1002 could be purified on an immobilized
249 metal affinity chromatography (IMAC) resin. The recombinant protein was eluted from
250 the resin at 150 mM imidazole, and observed as a single band on SDS-PAGE (**Figure 1**)
251 although some overproduced GroEL proteins were retained in the resin and eluted along
252 Lp_1002. The eluted protein was dialyzed against 50 mM phosphate buffer (pH 7)
253 containing 300 mM NaCl. Routinely about 20 mg of purified protein from 1-liter culture
254 was obtained. The calculated specific activity for the esterase Lp_1002 was 170 U/mg for
255 *p*-nitrophenyl acetate.

256 As esterases and lipases could not be distinguished by sequence comparison, the
257 activity of Lp_1002 against *p*-nitrophenyl esters possessing different acyl chain lengths
258 was assayed. Lp_1002 showed activity only on short-acyl chain esters, exhibiting a clear
259 preference for *p*-nitrophenyl acetate (**Figure 2A**), therefore confirming that Lp_1002 is a
260 true esterase.

261

262 **Substrate profile and biochemical characterization of Lp_1002.** Once the
263 esterase activity of Lp_1002 was confirmed, it was biochemically characterized in further
264 detail. To test the substrate range of esterases an ester library was used.³⁰ This ester library

consisted of esters that were chosen to identify acyl chain length preferences of the esterase and also the ability to hydrolyze charged substrates. From **Figure 2B** it can be deduced that Lp_1002 shows higher hydrolytic activity on phenyl acetate. Phenyl acetate is a commonly used substrate to identify bacterial arylesterases.⁶ Among esterases, arylesterases are of biotechnological interests since they are not only active toward aliphatic esters, but also towards aromatic ones, modulating the ester profile of food substrates. Arylesterases were less understood than other esterases, especially carboxylesterases or lipases.³¹ Until recently, most of the arylesterases reported were from mammalian sources, playing an important role in detoxification of organophosphorous compounds. In contrast, there are few reports on bacterial arylesterases, whose physiological function remains largely unknown. In addition, little is known about the genetic and biochemistry of bacterial arylesterases. Bacterial arylesterases have been only described from *Vibrio mimicus*,³² *Agrobacterium radiobacter*,³³ *Acinetobacter* sp.,³⁴ *Pseudomonas fluorescens*,³⁵ and *Sulfolobus solfataricus*.³¹ Among LAB, only arylesterases from *Lactobacillus helveticus*⁹ and *Lactobacillus casei*¹⁰ have been described. Finally, regarding wine-related bacteria, an arylesterase from *Gluconobacter oxydans* has been recently described.³⁶ As far as we know, Lp_1002 is the first arylesterase described in *L. plantarum* and in a wine LAB. Amino acid sequences of bacterial arylesterases showed less than 20% identity among them. The highest sequence identities detected were between Lp_1002 and *G. oxydans* (16% identity), *P. fluorescens* (16%), and *L. casei* (14%) arylesterases (**Figure 3**).

Bacterial arylesterases may be used for industrial purposes, especially considering the fact that they have broad substrate specificities.³² In this regard, although to a lesser extent than phenyl acetate, Lp_1002 also hydrolyze most of the substrates assayed (**Figure 2B**). Thus, Lp_1002 catalyzed the hydrolysis of small activated and small non-activated

esters with similar rates regardless of their size. Larger activated esters (e. g., vinyl decanoate) reacted similarly than larger non-activated esters (e.g., ethyl decanoate). Regarding the acyl chain selectivity, Lp_1002 exhibited hydrolytic activity towards all the ethyl esters, vinyl esters, and methyl esters assayed, possessing varied chain length as well as an electron-withdrawing group (e. g., ethyl bromoacetate and ethyl glycine) or an hindered group (e. g., vinyl benzoate and vinyl trimethylacetate). Considering the alcohol moiety selectivity, both acetates and butyrates were also hydrolyzed. From the 40 potential substrates assayed, only ethyl trifluoroacetate, chlorogenic acid and rosmarinic acid were not hydrolyzed by Lp_1002.

The broad substrate range exhibited by Lp_1002 indicated its potential utility in wine fermentations. From the substrates included in the ester library, relevant aroma compounds such as ethyl acetate (pineapple aroma), ethyl butanoate (floral, fruity), ethyl hexanoate (green apple), ethyl octanoate (sweet soap), and ethyl decanoate (floral, soap) were hydrolyzed by Lp_1002.³

Other important biochemical properties of Lp_1002 have been characterized. **Figure 4A** shows that the optimal temperature for Lp_1002 activity against phenyl acetate is 40 °C, retaining 50% of its maximal activity at winemaking temperatures (15-25 °C). Moreover, Lp_1002 showed thermal stability since 70% of its maximal activity was observed after 20 h incubation at 45°C or below (**Figure 4B**). The optimal temperature showed by Lp_1002 is similar to the optimal temperature exhibited by arylesterases from *P. fluorescens*,³⁰ *G. oxydans*²³ and *L. helveticus*,⁹ which are the arylesterases with the highest sequence similarity to Lp_1002. In addition, EstB28 and EstCOo8 esterases from a wine LAB, *O. oeni*, also showed optimal activity at 40 °C.¹³⁻¹⁴

Regarding the dependence on pH for Lp_1002 activity, **Figure 4C** showed that the protein was active between 3 and 8, exhibiting a broad optimal pH range (5 to 7). This

315 result indicates that Lp_1002 shows a marked preference for acidic conditions when
316 compared to other known bacterial arylesterases, which exhibited optimum pH values
317 ranging from 7 to 9. However, acidic esterases were previously described for esterases
318 from wine bacteria; thus, optimal pH around 5 was described for EstB28 and EstCOo8
319 from *O. oeni* and EstC34 from *L. hilgardii*.¹³⁻¹⁴ At pH values commonly found in wines
320 (pH 3-4), Lp_1002 still retained activity (20-40%), confirming its potential activity during
321 winemaking.

322 The effect of some metal ions and additives on Lp_1002 activity was studied and is
323 shown in **Table 1**. Esterase activity was significantly inhibited by Hg^{2+} , Cu^{2+} , and Ni^{2+}
324 ions, as well as by β -mercaptoethanol. The enzymatic activity was increased by Mn^{2+} ion
325 and by the detergents Tween-20 and Tween-80; contrarily, SDS inhibited 68% Lp_1002
326 activity. The increase in enzyme activity observed by non-ionic detergents, and the
327 decrease by an ionic detergent was previously described in an arylesterase isolated from
328 goat rumen³⁷ and in a feruloyl esterase from *L. plantarum*.²⁶

329
330 **Effect of wine compounds on Lp_1002 arylesterase activity.** The biochemical
331 properties showed by Lp_1002 indicated that the enzyme could be defined as an
332 arylesterase, potentially active at wine conditions. The observation that Lp_1002 is an
333 intracellular protein indicates that cell lysis may be important for its release and the
334 subsequent flavour formation during winemaking. Therefore, special attention should be
335 paid to the effect of compounds that could affect Lp_1002 activity once it has been
336 released to the media. With this aim, the influence of several compounds naturally present
337 in wine fermentations such as ethanol, sodium metabisulfite, and several organic acids,
338 such as malic, lactic, tartaric and citric acids, was studied.

Ethanol concentration is an important parameter during winemaking, since malolactic fermentation is often conducted after the completion of alcoholic fermentation, when the concentration of this compound in wine can exceed 12%.³⁸ For this reason, the influence of ethanol on the Lp_1002 arylesterase activity has been analyzed. Experiments were conducted with concentrations higher than those found in wines (up to 20%). Ethanol concentration up to 16% seems to increase Lp_1002 activity. The highest esterase activity was observed at ethanol concentration of 4% (**Figure 5A**). The activity of an esterase from the wine bacterium *O. oeni* was stimulated by 14% ethanol.¹³

In addition, sodium metabisulfite, a powerful antimicrobial commonly used in wine, did not inhibit the enzyme when present at concentrations commonly found in wine. Final levels of 0.1 to 0.2 g/l of metabisulfite are found in wines.³⁸ At wine concentrations, metabisulfite did not significantly affect Lp_1002 arylesterase activity (**Figure 5B**). In grape must there are several organic acids, such as malic acid (up to 8.6 g/L), or tartaric acid (up to 7.4 g/L). During vinification the concentration of these acids varies, and as a consequence of LAB metabolism, lactic acid appeared. In wines, concentrations of 5, 4 and 4 g/L (w/v) of malic, tartaric or lactic acid, respectively, could be found.³⁸ Esterase Lp_1002 retained high activity at wine concentrations of these organic acids (**Figure 5C-F**). Only 5 g/L malic acid concentrations inhibited 20% Lp_1002 activity (**Figure 5C**). The activity showed by Lp_1002 in the presence of wine compounds suggests that this arylesterase could play a role in modulating ester profiles during winemaking. The International Organization of Vine and Wine (OIV) recommends that side enzymatic activities which have a potential negative effect on wine should not be present in commercial enzyme preparations.³⁹ Therefore, the use of recombinant, pure, and widely biochemical characterized enzymes will avoid the negative side activities present on commercial preparations

364 In conclusion, in this work it is shown that Lp_1002 is the first arylesterase
365 described in *L. plantarum* and in a wine LAB. This arylesterase showed a broad substrate
366 range, being able to modify relevant compounds for wine aroma. Lp_1002 arylesterase
367 showed high activity under conditions commonly found during winemaking, such as cold
368 temperature, acid pH, the presence of ethanol, malic acid, tartaric acid, or sulphites. The
369 biochemical characteristics shown by Lp_1002 from *L. plantarum* suggests that this
370 arylesterase is a very promising enzyme during vinification

371

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375

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500

501 **FIGURE CAPTIONS**

502

503 **Figure 1.** Purification of Lp_1002 esterase. SDS-PAGE analysis of the expression and
504 purification of the His₆-Lp_1002 esterase. Analysis of soluble cell extracts of IPTG-
505 induced *E. coli* BL21(DE3) (pURI3-Cter) (pGro7) (1) or *E. coli* BL21(DE3) (pURI3-Cter-
506 1002) (pGro7) (2), flowthrough (3), or eluted protein after His affinity resin (4). The arrow
507 indicated the overproduced and purified protein. The gel was stained with Coomassie blue.
508 Molecular mass markers are located at the left (SDS-PAGE Standards, Bio-Rad).

509

Figure 2. Substrate profile of Lp_1002 against chromogenic substrates (*p*-nitrophenyl esters) with different acyl chain lengths (C2, acetate; C4, butyrate; C8, caprylate; C12, laurate; C14, myristate; C16, palmitate) (A) or toward a general ester library (B). The relative activities obtained toward different substrates are shown. The error bars represent the standard deviation estimated from the three independent assays. The observed maximum activity was defined as 100%.

Figure 3. Comparison of amino acid sequences of bacterial arylesterases from *Lactobacillus casei* (LCA), *Gluconobacter oxydans* (GOX), *Lactobacillus plantarum* (LPL), and *Pseudomonas fluorescens* (PFL). Multiple alignments were done using the program ClustalW2 after retrieval of sequences from BLAST homology searches. Residues that are identical (*), conserved (:), or semiconserved (.) in all sequences are indicated. Dashes indicated gaps introduced to maximize similarities.

Figure 4. Biochemical properties of Lp_1002 esterase. (A) Relative activity of Lp_1002 versus temperature. (B) Relative activity versus pH. (C) Thermal stability of Lp_1002 after preincubation at 22 °C (filled diamond), 30 °C (filled square), 37 °C (filled triangle), 45 °C (cross), 55 °C (star), and 65 °C (filled circle) in phosphate buffer (50 mM, pH 7); at indicated times, aliquots were withdrawn, and analyzed as described in the Methods section. The experiments were done in triplicate. The mean value and the standard error are shown. The observed maximum activity was defined as 100%.

Figure 5. Activity of Lp_1002 esterase in the presence of compounds frequently found in wine. Relative activity of Lp_1002 after incubation in the presence of ethanol (A), sodium metabisulfite (B), malic acid (C), lactic acid (D), tartaric acid (E), and citric acid (F) at the

535 concentrations indicated. The activity of the enzyme in the absence of the compound was
536 defined as 100%. The experiments were done in triplicate. The mean value and the
537 standard error are shown. Asterisks indicate a P value <0.05 .

Table 1

Table 1. Effect of additives on Lp_1002 esterase activity

Additions (1 mM)	Relative activity (%)
Control	100
KCl	105
HgCl ₂	14
CaCl ₂	125
MgCl ₂	80
ZnCl ₂	58
CuCl ₂	13
NiCl ₂	23
MnCl ₂	129
Tween 20	116
Tween 80	115
Triton-X-100	122
SDS	32
Urea	97
DMSO	106
Cysteine	117
DTT	92
β-mercaptoethanol	16
EDTA	98
PMSF	65
DEPC	105

Figure 1

Figure 2

Figure 3

LCA	-----MADDDILAKIQAGWAQTAARDKARYADERVPEDVHWETEYRYE	43
GOX	MNTRSLVLPPELLPLLGEIPTVDLSAESLPAFRAGLEQTAGQSFLQAEDYPVTLERLNIPG	60
LPL	-----MQVIKQKL	8
PFL	-----MSTFVAKDGTQIYFKDW	17
	:	
LCA	NSADPQQTNLNLYYPAKRRNATLPTVIDVHGGGWFGDRNLN-RNYCRYLASQGYAVMGMG	102
GOX	TENAPP I RLLTVRPRKEATGPRRALLHLHGGGYFAGQPELTTTQLCQFAQELDCVVISPD	120
LPL	TATCAQLTGYLHQPDTHAQTNLPAIIIVPGGSYTHIPVAQAESLAMAFAGHGYQAFYLE	68
PFL	GSGKPVLFSHGWLDDADMWEYQMEYLSSR-GYRTIAFDRRGFGRSDQPWTGNDYDTFADD	76
	: *	
LCA	YRLLPDVDVR--GQIQDIFASLRWLSHFQPGQGFDLDHVLLTGDSAGGHLASLVACIQQS	160
GOX	YRLTPEHPFP--AAIEDAYTTLCWITRNADTLGIDPARIGLTGESAGGGLAAGLALLTRD	178
LPL	YTLLTDQQPLGLAPVLDLGRAVNLLRQHAAEWHIDPQQITPAGFSVGGHIVALYNDYWAT	128
PFL	IAQLIEHLDLKEVTLVGFSMGGGDVARYIARHGSAR----VAGLVLLGAVTPLFGQKPDY	132
	: : *	
LCA	EELQELFGVDRVN----FNFTLVALVCPVAEPGKLPEAAGDMSDMAAFYLDKLSGGDAALA	217
GOX	REGPALLFQNL IYPVLDDRTITGQPDASPVGGEFLWTRASNTFMWTALLNPVAPGSADVS	238
LPL	RVATELNVTPAMLK--PNNVVLGYPVISPLLG-FPKDDATLATWTPTPNELAADQHVNSD	185
PFL	PQGVPLDVFAFVKT--ELLKDRAQFISDFNAPFYGINKGQVVSQGVQTQLQIALLASLK	190
	* :	
LCA	DHLNFSQVAKDLKLPPFMLIGGQNSFYLQSQALLEVF AADKVTTYTTKLWPASAGPHLKH	277
GOX	PYAAPARATDLSDLPPVYLCVGALDLFLDEDLAYARRLIHQGISVELEIVPG-----	290
LPL	NQPTFIWTTADDP IVP-----ATNTLAYATALATAKIPYELHVFKHG-----PH	229
PFL	ATVDCVTAF AETDFRP-----DMAKIDVPTLV IHGDGDQIVPFETTG---KVAA	236
	: *	
LCA	VFNVQHWEWPES IETNLAMLRQFDVLSKQHDEAEADEEDDL	318
GOX	--ACHIFDQTDTPVATRSIARRIDAMKRGFGL-----	320
LPL	GLALANAQTAWKPDANQPHVAHWLTLALEWLADNR-----	264
PFL	ELIKGAELKVYKDAPHGFAVTHAQQLNEDLLAFLKR-----	272
	: :	

Figure 4

Figure 5