Characterization of a cold-active esterase from *Lactobacillus plantarum* suitable for food fermentations

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Abstract

Lactobacillus plantarum is a lactic acid bacteria that can be found in numerous fermented foods. Esterases from *L. plantarum* exert a fundamental role in food aroma. In the present study, the gene *lp_2631* encoding a putative esterase was cloned and expressed in *Escherichia coli* BL21 (DE3) and the overproduced Lp_2631 protein has been biochemically characterized. Lp_2631 exhibited optimal esterase activity at 20 ºC and more than 90% of maximal activity at 5 ºC, being the first cold-active esterase described in a lactic acid bacteria. Lp_2631 exhibited 40% of its maximal activity after 2h incubation at 65 ºC. Lp_2631 also showed marked activity in the presence of compounds commonly found in food fermentations, such as NaCl, ethanol, or lactic acid. The results suggest that Lp_2631 might be a useful esterase to be used in food fermentations.

KEYWORDS: Esterase, Food fermentation, Aroma, Esters, Lactic acid bacteria

INTRODUCTION

In fermented foods, microorganisms are in contact with food substrates, and their metabolic activities influence food aroma.[1-2] Esters are important in determining the aroma, and, by extension, the quality of foods. These compounds are formed when alcohol and carboxylic acid functional groups react, and a water molecule is eliminated. Enzymatic ester synthesis and hydrolysis are catalyzed by esterases.[3] Since lactic acid bacteria (LAB) are extensively used for the fermentation of food products, LAB esterases could influence the aroma of these fermented products.[4-9] Among LAB, *Lactobacillus plantarum* is an industrially important species which can be found in numerous fermented foods, such as sourdoughs, olives, vegetables, sausages, cheese, and wine.[10] *L. plantarum* is a good source of esterase enzymes since lipolytic and esterase activity have been previously described in *L. plantarum* strains. Although esterases or lipases are common in *L. plantarum*, so far only few have been partially purified, purified or recombinantly produced.[6, 11-19] These *L. plantarum* proteins exhibited different esterase activities and biochemical properties, such as lipases,[6,11-16] acetyl esterases, [17] or feruloyl esterase.[18]

So far, all the esterases described in *L. plantarum* exhibited optimal temperature for activity around 30-40 ºC. However, the role of *L. plantarum* in aroma development could be important when optimal activity conditions are close to those found in meat, milk or wine fermentations. During these fermentations temperatures as low as 15 ºC are found. For most of the enzymes, lowering the temperature by 10 ºC decreases the rate of reaction by two to three fold.[19] Currently, cold-adapted esterases have emerged as one of the most promising biocatalysts because, when compared with mesophilic or thermophilic enzymes, they display a much higher catalytic efficiency at a low or moderate temperature. This feature can meet the demands of some industrial applications, including additives in food

processes (fermentation, cheese manufacture, bakery, meat tenderizing). So far, only a few cold-adapted esterases have been discovered and characterized, [19] none of them from LAB.

As *L. plantarum* is a mesophilic organism which would often encounter temperatures below 20 ºC in nature or in food fermentations, it seems likely that it would be a good source for enzymes with interesting novel properties, e. g., enzymes active in cold conditions. The genome sequence of *L. plantarum* WCFS1 was published in 2003 [10] and more than twenty putative esterase or lipase genes were annotated on the basis of similarity searches. Although an operational distinction is made between esterases, which preferentially break the ester bonds of shorter chain acyl substrates at least partly soluble in water, and lipases, which display maximal activity toward water-insoluble long-chain triglycerides, there is no fundamental biochemical difference.[3] Both esterases and lipases are members of the α/β hydrolase superfamily, and share the same catalytic mechanisms for ester hydrolysis and formation.[3] Classifications based on sequence similarities do not separate the two classes of enzymes.

This study presents an effort to elucidate the ester hydrolysis activities in *L. plantarum* whole cells by cloning, heterologous expression, purifying and characterizing the esterase Lp_2631 from *L. plantarum*. Esterase Lp_2631 is the first cold-active esterase described in LAB species used in food fermentations. With a view to applying Lp_2631 cold-active esterase under fermentation conditions, enzyme activity in the presence of compounds commonly found in food fermentations was examined.

MATERIALS AND METHODS

Strains, plasmids, media and materials. *L. plantarum* WCFS1, kindly provided by M. Kleerebezem (NIZO Food Research, The Netherlands), was grown in MRS medium (Pronadisa, Spain) adjusted to pH 6.5 and incubated at 30 ºC. This strain is a colony isolate of *L. plantarum* NCIMB 8826, which was isolated from human saliva. *Escherichia coli* DH10B was used as host strain for all DNA manipulations. *E. coli* BL21 (DE3) was used for heterologous expression in the pURI3-TEV vector.[21] *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37 ºC and shaking at 200 rpm. When required, ampicillin and chloramphenicol were added to the medium at a concentration of 100 or 20 μg/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). Oligonucelotides were purchased from Eurofins MWG Operon (Ebersberg, Germany). *Dpn*I and HS Prime Star DNA polymerase were obtained from TaKaRa. His-tagged protein was purified by a Talon Superflow resin (Clontech).

Cloning of Lp_2631 esterase encoding gene. Genomic DNA from *L. plantarum* WCFS1 was extracted. The gene encoding a putative lipase/esterase (*lp_2631*) in *L. plantarum* WCFS1 was amplified by PCR by using the primers 569 (5´-*GGTGAAAACCTGTATTTCCAGGGCatg*gtgcggacgaccaaatttggtg) and 570 (5´-*ATCGATAAGCTTAGTTAGCTATTATTA*tttaagaatgttggtctccaag) (the nucleotides pairing the expression vector sequence are indicated in italics, and the nucleotides pairing the *lp_2631* gene sequence are written in lowercase letters). Prime Star HS DNA polymerase (TaKaRa) was used for the PCR amplification. The 786-bp purified PCR product was inserted into the pURI3-TEV vector using a restriction enzyme- and ligation-free cloning strategy.[21] The vector produces recombinant proteins having a six-histidine affinity tag in

115 their N-termini. *E. coli* DH10B cells were transformed, recombinant plasmids were
116 isolated, and those containing the correct insert were identified by size, verified by DNA
117 sequencing, and then transformed into *E. coli* BL21 (DE3) cells for expression.
118
119 **Expression and purification of Lp_2631 esterase**. *E. coli* BL21(DE3) harbouring
120 pGro7 (TaKaRa), a vector overexpressing GroES/GroEL chaperones, was transformed
121 with the recombinant plasmid pURI3-TEV-2631. *E. coli* was grown in LB medium
122 containing 100 μg/mL ampicillin, 20 μg/mL chloramphenicol, and 2 mg/mL arabinose, on
123 a rotary shaker (200 rpm) at 37 ºC until an optical density (OD) at 600 nm of 0.4 was
124 reached. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration
125 of 0.4 mM and protein induction was continued at 22 ºC during 18 h.
126 The induced cells were harvested by centrifugation (8,000 *g*, 15 min, 4 ºC),
127 resuspended in phosphate buffer (50 mM, pH 7) containing 300 mM NaCl and disrupted
128 by French Press passages (three times at 1,100 psi). The insoluble fraction of the lysate
129 was removed by centrifugation at 47,000 *g* for 30 min at 4 ºC, and the supernatant was
130 filtered through a 0.2 μm pore-size filter and then loaded onto a Talon Superflow resin
131 (Clontech) equilibrated in phosphate buffer (50 mM, pH 7) containing 300 mM NaCl and
132 10 mM imidazole to improve the interaction specificity in the affinity chromatography
133 step. The bound enzyme was eluted using McIlvaine buffer (100 mM, pH 5).[22] The purity
134 of the enzyme was determined by SDS-PAGE in Tris-glycine buffer. Fractions containing
135 the His6-tagged protein were pooled and analyzed for esterase activity.
136
137 **Enzyme assay.** Esterase activity was determined by a spectrophotometric method as
138 described previously using *p*-nitrophenyl acetate (Sigma-Aldrich) as the substrate.[18]
139

Substrate specificity. To investigate the substrate specificity of Lp_2631, activity was determined using different *p*-nitrophenyl esters of various chain lengths (Sigma-Aldrich): *p*-nitrophenyl acetate (C2), *p*-nitrophenyl butyrate (C4), *p*-nitrophenyl caprylate (C8), *p*-nitrophenyl laurate (C12), *p*-nitrophenyl myristate (C14) and *p*-nitrophenyl palmitate (C16) as substrates as described previously.[18, 23]

Determination of optimum pH, temperature and thermostability. Effect of pH was studied by assaying esterase activity in a range of pH values from 3.0 to 9.0 as described previously.[18] Temperature effect was assayed in 50 mM sodium phosphate buffer (pH 7.0) at 5, 20, 30, 37, 40, 45, 55, and 65 ºC for 10 min. For temperature stability measurements, the esterase was incubated in 50 mM sodium phosphate buffer (pH 7.0) at 22, 30, 37, 45, 55 and 65 ºC for 5 min, 15 min, 30 min, and 1, 2, 4, 6, and 20 h. After incubation, the residual activity was measured as described above.

Effects of additives on Lp_2631 esterase activity. The effect of metals ions, surfactants, reductants, and inhibitors on the activity of the esterase was assayed by incubation of the enzyme in the presence of different additives at a final concentration of 1 mM during 5 min at room temperature. Then, the substrate was added and the reaction was incubated at 20 ºC.[18] The compounds analyzed were $MgCl_2$, KCl, $MnCl_2$, $CuCl_2$, $NiCl_2$, $CaCl_2$, $HgCl_2$, $ZnCl_2$, diethylpyrocarbonate (DEPC), Cysteine, SDS, DTT, Triton-X-100, Urea, Tween 80, Tween 20, ethylenediamine tetracetic acid (EDTA), dimethyl sulfoxide (DMSO), phenylmethanesulfonyl fluoride (PMSF) and β-mercaptoethanol.

In addition, the effect of several compounds present in food fermentation 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 (1 mL). 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. Malic, tartaric, citric, and 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) prepared in phosphate buffer (50 mM, pH 7.0). 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. Finally, the effect of NaCl was determined by adding NaCl at concentrations ranging from 0 to 25% (vol/vol). Reaction mixtures were prepared as described for the temperature optimum experiments, but different volumes of 25% NaCl solution were added, and the volume of the buffer was adjusted accordingly to maintain the final reaction volume. Reactions mixtures were pre-incubated for 5 min at room temperature before the enzyme was added. After the reaction, the absorbance was measured at 348 nm.

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

RESULTS AND DISCUSSION

Production and characterization of Lp_2631 esterase. LAB are used for the preservation of food raw materials such as milk, meat, and vegetable or other plant

materials. *L. plantarum* is a flexible and versatile species that is encountered in a variety of environmental niches, including some dairy, meat, and many vegetable or plant fermentations. The ecological flexibility of *L. plantarum* is reflected by the observation that this species has one of the largest genomes known among LAB.[10] This large genome codifies enzymatic activities which could develop a fundamental role in food fermentations, such as cold-active esterases (also known as cold-adapted). When the published sequence of *L. plantarum* WCFS1 was analyzed, numerous ORFs encoding putative esterases/lipases were found. As it is not possible predict the biochemical function encoded by these *L. plantarum* ORFs, features found in cold-active esterases were searched. According to previous studies,[24-26] compared with mesophilic or themophilic esterases, cold-adapted enzymes revealed structural traits, such as a low ratio or Arg/(Arg+Lys), a low proportion of proline residues, or a high proportion of small residues (Gly and Ala). Among the putative esterases encoded by the *L. plantarum* WCFS1 genome, *lp_2631* encodes a protein that exhibits some of these structural features. Thus, when the amino acid composition of Lp_2631 was analyzed, the ratio of Arg/(Arg+Lys) was found to be 0.45, lower than that of other cold-adapted esterases: 0.50 in Est10 from *Psychrobacter pacifiensis*,[27] or 0.56 in rESt97 from a metagenomic library.[28] Also the total percentage (20.31%) of small residues Gly (7.28%) and Ala (13.03%) is relatively high compared with the low proportion of Arg (3.45%) and Pro (5.75%).These characteristics of the primary structure may suggest that Lp_2631 could be a cold-active esterase.

Lp_2631 is predicted to encode a 261 amino acid protein 42% identical to a putative esterase/lipase from *Lactobacillus sakei*, and 35% identical to uncharacterized proteins from *Lactobacillus salivarius* or *Lactobacillus ruminis* (data not shown).

The *lp_2631* gene was cloned into the pURI3-TEV expression vector and the recombinant plasmid obtained was transformed into *E. coli* BL21 (DE3). Cell extracts were used to detect the presence of overproduced proteins. SDS-PAGE analysis showed that there was one major band of protein, approximately 28 kDa, present as inclusion bodies in the insoluble fraction. In order to get Lp_2631 soluble, plasmid pGro7, producing GroES/GroEL chaperones, was used. When pURI3-TEV-2631 and pGro7 plasmids were used simultaneously, Lp_2631 appeared in the intracellular soluble fraction of the cells (**Figure 1**). The molecular weight of the overproduced protein was consistent with the theoretical one expected for Lp_2631. The recombinant protein was observed as single band on SDS-PAGE (**Figure 1**). Routinely about 8 mg of purified protein from 1-liter culture was obtained.

Lp_2631 protein purified by the affinity resin was biochemically characterized. Substrate specificity was determined using *p*-nitrophenyl-linked esters of various acyl chain lengths (C2 to C16) at 20 ºC (**Figure 2**). The enzyme was only active on substrates with a chain length up to C4, with minimal activity detected with the longer chain lengths. This result indicated that Lp_2631 is an esterase and not a true lipase. This narrow substrate range displayed by Lp_2631 is similar to the substrate range described for a *L. plantarum* esterase described previously.[23]

The influence of pH, in the range 3.0 to 9.0, on esterase activity was studied at 20 ºC (**Figure 3A**). Although the enzyme showed activity at pH from 3.0 to 8.0, the highest activity was observed at pH 6.5. Similarly to the substrate range, only the esterase described previously by Brod et al (2010) exhibited an optimal pH range of 6.0-6.5. Apart from these both esterases, the *L. plantarum* esterases described so far showed higher optimum pH, 7 for Lp_2923 [29] and a feruloyl esterase, [18] and pH 8 for Lp_0796.[30] LAB fermented food products are characterized by a low pH. For example, a final pH around

239 5.5 could be found in meat fermentations; at this pH, Lp_2631 still exhibited 40% of its
240 maximal activity.

241 The influence of temperature on enzymatic activity was determined (**Figure 3B**).
242 Maximal activity was observed at 20 ºC, confirming previous structural data of Lp_2631
243 suggesting that this esterase could be a cold-adapted protein. As far as we know, Lp_2631
244 is the first esterase from *L. plantarum* exhibiting such functional features. In fact, other *L.*
245 *plantarum* esterases show higher optimal temperatures, such as 30 ºC for Lp_2923,[29] 35
246 ºC for Lp_0796, [30] or 40 ºC for Lp_0973.[31] More interestingly, Lp_2631 showed more
247 than 90% activity at 5 ºC, and decreases to 30% at 30 ºC. Similar behaviour has been
248 described in cold-active esterases described from non-LAB or metagenomic libraries.[31-34]
249 Lp_2631 is the first esterase described from LAB which shows higher activity at
250 refrigeration temperatures. This is an important property in food fermentations; e. g.,
251 during the ripening of a fermented meat product in the traditional manufacturing process,
252 temperatures can be as low as 15 ºC; moreover esterase activity could be important even at
253 meat storage temperatures (3 ºC, 7.5 ºC).[33]

254 The available data regarding psychrophilic enzymes pointed out that the high
255 specific activity at low temperatures is often associated to a low thermostability, which
256 make them inclined to loose their activity at moderate and even high temperature.[24] Low
257 temperature activity has been generally associated with low conformational stability. In
258 order to assess the thermostability of Lp_2631, the enzyme was preincubated at different
259 temperatures for different time intervals, before assaying the residual activity. **Figure 3C**
260 shows that Lp_2631 was fairly stable under room temperatures. The enzyme retained up to
261 80% activity after incubation during 20 h at 20 ºC. The enzyme showed 50% of its
262 maximal activity after 4 h incubation at 30 or 37 ºC, or 40% after 2h at 45 to 65ºC.
263 Therefore, esterase Lp_2631 not only showed high activity levels at low temperature, but

264 also exhibited higher thermostability when compared with other cold-active enzymes, e.g.,
265 the low-temperature lipase from psychrotopic *Pseudomonas* sp. strain KB700A.[36] In fact,
266 other cold-adapted esterases are also fairly stables.[37] Although high catalytic activity at
267 low temperature tends to be associated with thermosensitivity, directed evolution studies
268 to improve the thermostability of cold-adapted enzymes revealed that, as in Lp_2631,
269 there is not a strict correlation.[19]

270 The effects of several ions, surfactants, reductants, and inhibitors on Lp_2631
271 activity are shown in **Figure 4**. Compared to the enzyme incubated in 50 mM phosphate
272 buffer pH 7, the enzymatic activity was increased two-fold by the detergents Tween-20
273 and Tween-80, and, by contrast, SDS greatly inhibited Lp_2631. The increase in enzyme
274 activity observed by non-ionic detergents, and the decrease by ionic detergent was
275 previously described in a feruloyl esterase from *L. plantarum* [16] and in the cold-active
276 lipase EML1 from a deep-sea sediment metagenome.[38] Regarding to metal ions, only
277 Mn^{2+} increases activity, while Ni^{2+}, Cu^{2+}, and Hg^{2+} significantly inhibited esterase activity.
278 Inactivation by Hg^{2+} ions has been previously described in other *L. plantarum* esterases,
279 such a feruloyl esterase [18] and a lipase purified from *L. plantarum* 2739.[14] Normally, the
280 esterase activity of hydrolases does not require cofactors such as metals. On the contrary,
281 the inhibitory effects of some of then are observed and explained in terms of covalent
282 modification of catalytic residues (such as the covalent modification of thiol groups by
283 Hg^{2+}). The effects of other metals on esterase activity (either activating or inhibiting the
284 enzyme) have also been reported; [29] however, the molecular mechanisms underlying these
285 effects have not been determined and most probably they are unspecific. The enzyme was
286 also partially inactivated by PMSF and DEPC confirming the involvement of a serine and
287 a histidine at the active site of the enzyme.
288

289 **Activity of Lp_2631 in the presence of compounds found in food**
290 **fermentations.** Also important, in order to understand the role of Lp_2631 esterase
291 activity during food processing, is the study of compounds present in food fermentation
292 media that could affect its activity. Food processing subjects microorganisms, and their
293 enzymes, to adverse environmental conditions (such as osmotic stress, toxic compounds,
294 ethanol), which affect their survival and technological performances. Because of its
295 industrial relevance, a better understanding of the influence of compounds present in food
296 fermentations is important. The influence on Lp_2631 activity of several compounds
297 naturally present in food fermentations such as ethanol, malic acid, tartaric acid, and
298 sodium metabisulfite in winemaking, NaCl in cheese and meat fermentations, and lactic
299 acid as a main product resulting for LAB fermentation was also studied.
300 Ethanol concentration is an important compound during winemaking, because
301 malolactic fermentation is often conducted after the completion of alcoholic fermentation,
302 when ethanol concentration of wine can exceed 12%.[39] For this reason, the influence of
303 ethanol on the Lp_2631 esterase activity has been analyzed. Experiments were conducted
304 with concentrations higher than those found in wines (up to 20%). The highest esterase
305 activity was observed in absence of ethanol, however, at wine ethanol concentrations
306 (12%), Lp_2631 exhibited a 40% of its maximal activity (**Figure 5A**). A different ethanol
307 effect was observed in an esterase from the wine bacterium *O. oeni* since it was stimulated
308 by 14% ethanol, producing a 49% activity increase.[22]
309 In addition, sodium metabisulfite, a powerful antimicrobial commonly used in
310 wine, did not inhibit the enzyme when present at concentrations commonly found in wine.
311 Final levels of 0.1 to 0.2 g/L of metabisulfite are found in wines.[39] At wine concentrations,
312 metabisulfite increases up to 70% esterase activity (**Figure 5B**). Again, Lp_2631 esterase
313 showed a different behaviour than *O. oeni* esterase which was partially inhibted in

314 presence of metabisulfite.[22] In grape must there are several organic acids, such as malic
315 acid (up to 8.6 g/L), or tartaric acid (up to 7.4 g/L). During vinification the concentration
316 of these acids varies, and as a consequence of LAB metabolism, lactic acid appeared. In
317 wines, concentrations of 5, 4 and 4 g/L of malic, tartaric or lactic acid, respectively, could
318 be found.[39] Esterase Lp_2631 showed activity at wine concentrations of these organic
319 acids. Malic acid concentrations up to 5 g/L increased esterase activity (**Figure 5C**);
320 however, esterase was partially inhibited by tartaric and lactic acid at wine concentrations
321 (**Figure 5D and 5E**). Lp_2631 activity was not affected by concentrations of citric acid
322 usually found in grape musts or wines, maximum 0.90 and 0.88 g//L, respectively.[37]
323 *L. plantarum* strains are active in fermented foods, such as milk or meat
324 fermentations, in which salt, citric or lactic acid are present.[40] Under cheese ripening
325 conditions (4% NaCl), Lp_2631 was only partially inhibited, retained 74% of its maximal
326 activity at 5% (**Figure 5G**). Citric and lactic acid showed a similar behaviour, both acids
327 inhibited Lp_2631 activity at 5 g/L, whereas at 1 g/L the enzyme keeps full activity. At
328 cheese concentrations (50 mg/100 g) [41], citric acid did not inhibit Lp_2631 activity. The
329 activity showed by Lp_2631 suggests that this esterase could play a role in modulating
330 ester profiles during cheese ripening.
331 The obtained results indicated that Lp_2631 is an esterase which retains activity
332 under conditions commonly found in food fermentations, such as cold temperature, the
333 presence of salt, ethanol, organic acids, or antimicrobials. The main disadvantage for the
334 use of Lp_2631 in food fermentations is its narrow pH profile under standard reaction
335 conditions. Further assays need to be done under combined conditions in order to known
336 the effect of the simultaneous presence of different factors on Lp_2631 activity.
337

In conclusion, in the present study the cold-active esterase namely Lp_2631 from *L. plantarum*, an industrially important LAB species which can be found in numerous fermented foods, was purified and biochemically characterized. Lp_2631 is a cold-active esterase that exhibited maximum activity at 20 ºC, and more importantly, retains more than 90% activity at refrigeration temperatures. In addition, esterase Lp_2631 not only showed a good activity at low temperatures, but also had good thermostability compared with other cold-active enzymes. Based on the findings reported in this study, it appears that Lp_2631 will retain adequate activity under food fermentation conditions.

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FIGURE CAPTIONS

Figure 1. SDS-PAGE analysis of the purification of Lp_2631 esterase from *L. plantarum* WCFS1. Analysis by SDS-PAGE of soluble cell extracts of IPTG-induced *E. coli* BL21(DE3) (pGro7)(pURI3-TEV) (1) or *E. coli* BL21(DE3) (pGro7) (pURI3-TEV-2631) (2), flowthrough (3), or protein eluted after His affinity resin (4). The gel was stained with Coomassie blue. Molecular mass markers are located at the left (SDS-PAGE Standards, Bio-Rad).

484

485 **Figure 2**. Substrate profile of Lp_2631 against chromogenic substrates (*p*-nitrophenyl
486 esters) with different acyl chain lengths (C2, acetate, C4, butyrate, C8, caprylate, C12,
487 laurate, C14, myristate, C16, palmitate). The figure displays the relative specificities
488 obtained toward different substrates, and lines on top of each bar represent the standard
489 deviations estimated from three independent assays. The observed maximum activity was
490 defined as 100%.

491

492 **Figure 3**. Biochemical properties of Lp_2631 esterase. (A) pH-activity profile of
493 Lp_2631. (B) Temperature-activity profile of Lp_2631. (C) Thermal stability profile for
494 Lp_2631 after preincubation at 22 ºC (circle), 30 ºC (square), 37 ºC (diamond), 45 ºC
495 (triangle up), 55 ºC (triangle down), and 65 ºC (star) in phosphate buffer (50 mM, pH 7),
496 at indicated times, aliquots were withdrawn, and analyzed as described in the Materials
497 and Methods section. The experiments were done in triplicate. The mean value and the
498 standard error are showed. The percentage of residual activity was calculated by
499 comparing with unincubated enzyme.

500

501 **Figure 4**. Effects of additives on Lp_2631 esterase activity. Relative activity of Lp_2631
502 after incubation with 1mM concentration of different additives. The activity of the enzyme
503 in the absence of additives was defined as 100%. The experiments were done in triplicate.
504 The mean value and the standard error are shown.

505

506 **Figure 5.** Activity of Lp_2631 esterase in the presence of compounds found in food
507 fermentations. Relative activity of Lp_2631 after incubation in the presence of compounds
508 present in food fermentation media such ethanol (A), sodium metabisulfite (B), malic acid

509 (C), tartaric acid (D), lactic acid (E), citric acid (F), and NaCl (G) at the concentrations
510 indicated. The activity of the enzyme in the absence of the compound was defined as
511 100%. The experiments were done in triplicate. The mean value and the standard error are
512 shown. Asterisks indicate a P value <0.05.
513

Figure 1

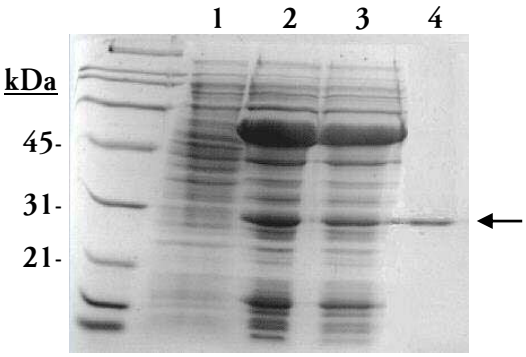
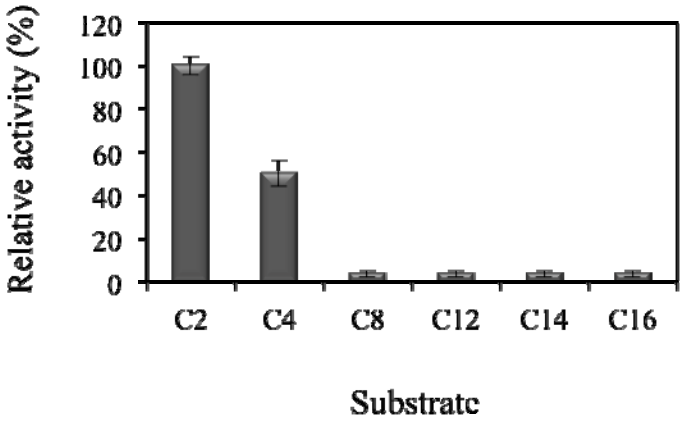
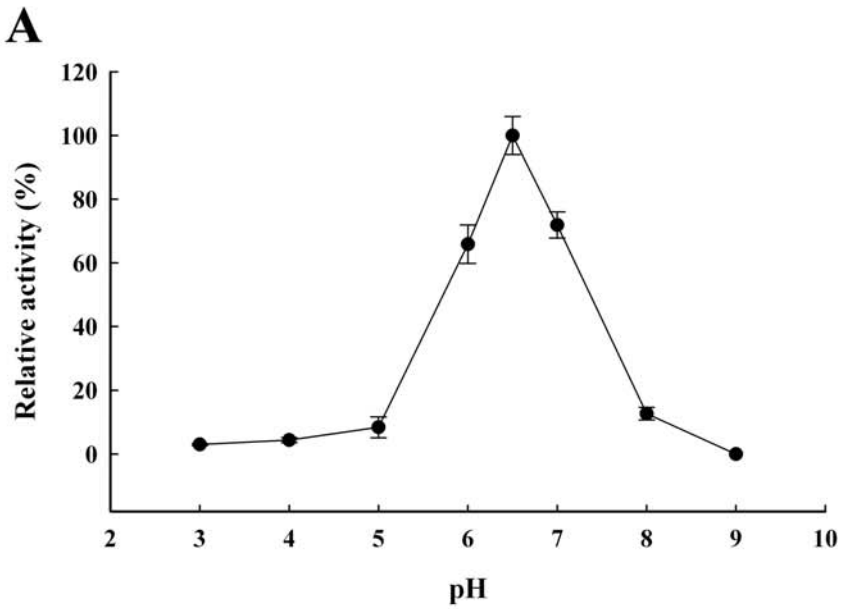
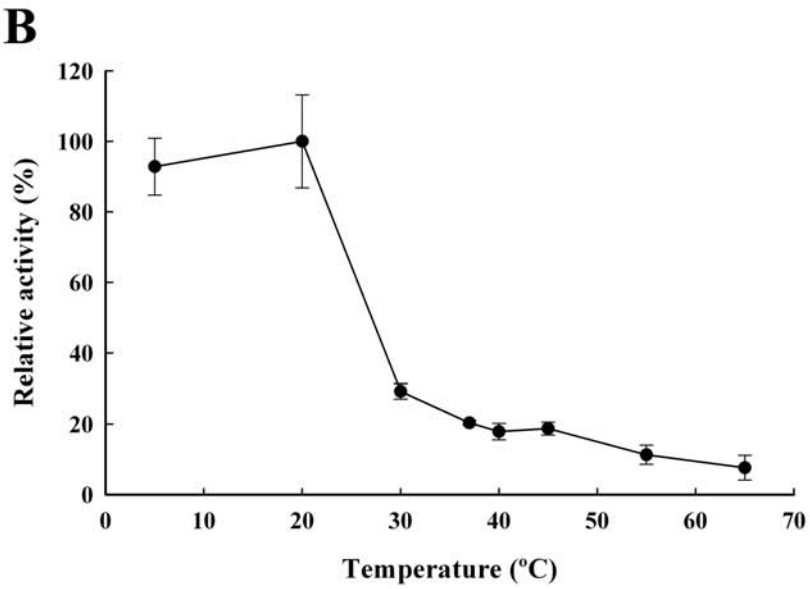


Figure 2


Relative activity (%)
120
100
80
60
40
20
0
C2
C4
C8
C12
C14
C16
Substrate


A
Relative activity (%)
120
100
80
60
40
20
0
2
3
4
5
6
7
8
9
10
pH


B
Relative activity (%)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
Temperature (°C)

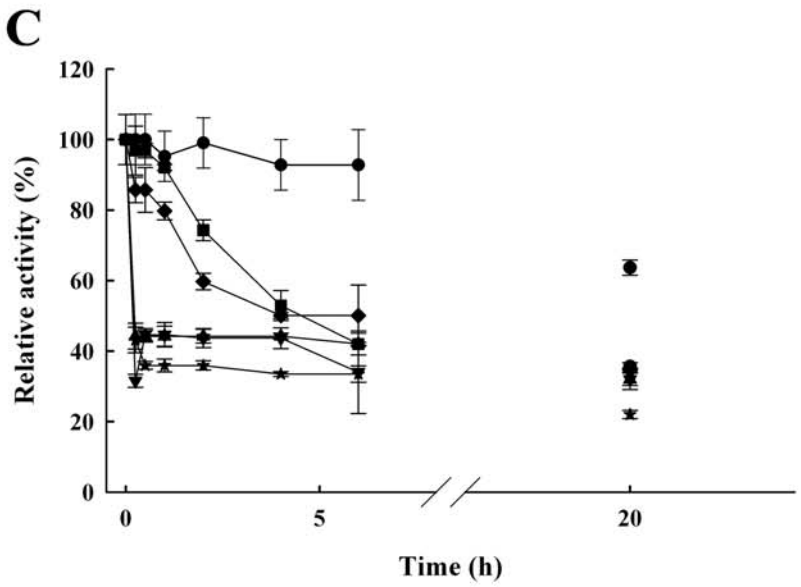
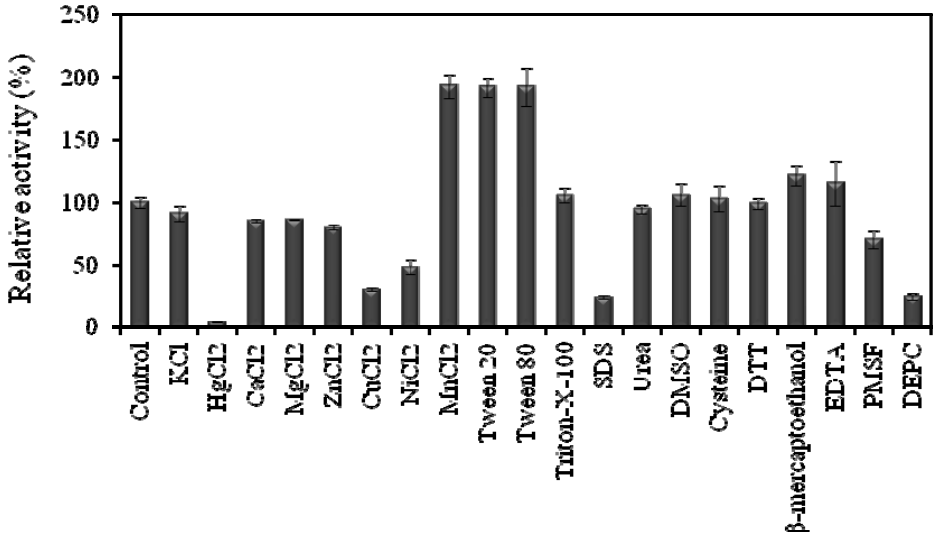
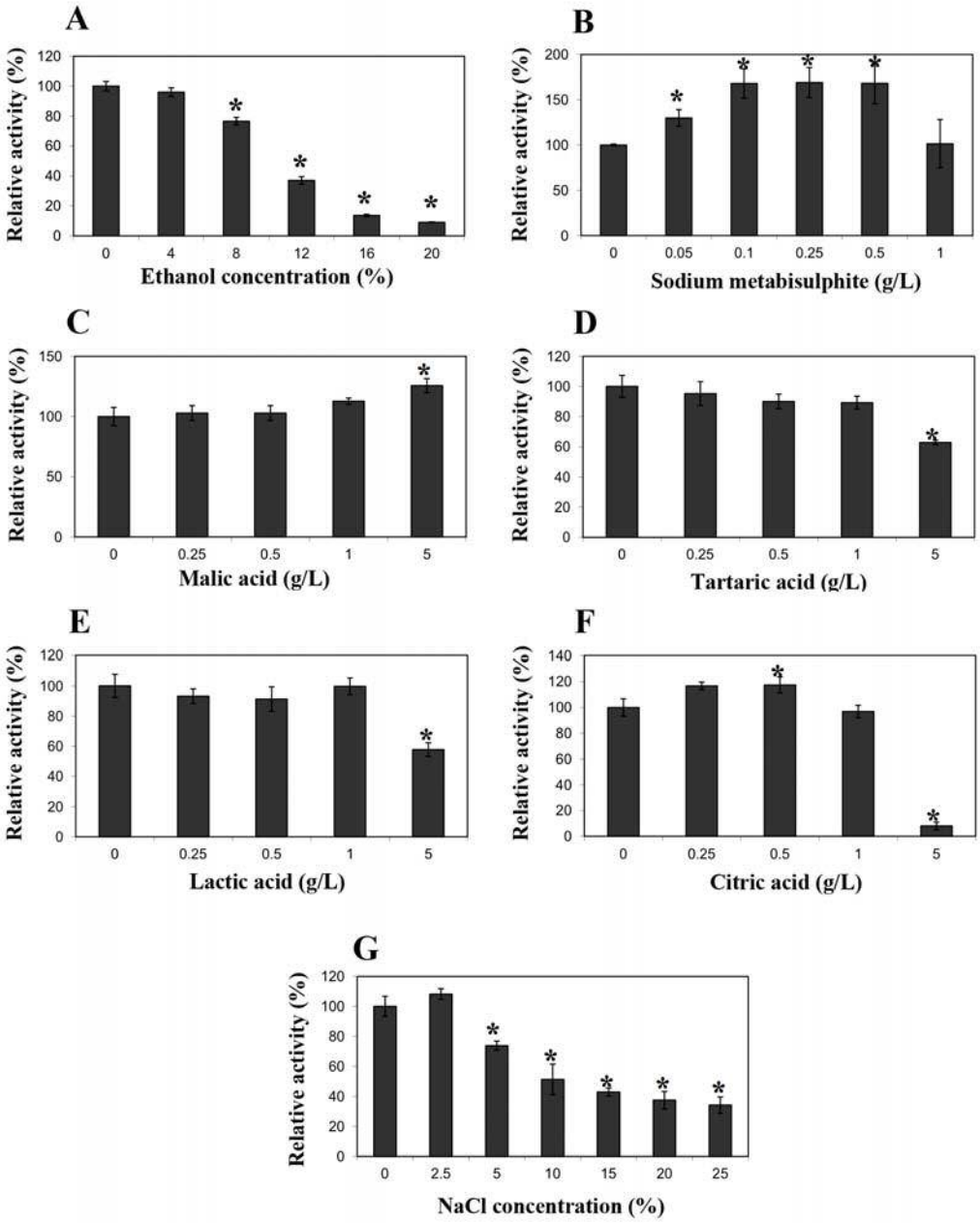

C
Relative activity (%)
120
100
80
60
40
20
0
0
5
20
Time (h)

Figure 4


Relative activity (%)
250
200
150
100
50
0
Control
KCl
HgCl2
CaCl2
MgCl2
ZnCl2
CuCl2
NiCl2
MnCl2
Tween 20
Tween 80
Triton-X-100
SDS
Urea
DMSO
Cysteine
DTT
β-mercaptoethanol
EDTA
PMSF
DEPC


A
Relative activity (%)
120
100
80
60
40
20
0
0
4
8
12
16
20
Ethanol concentration (%)
B
Relative activity (%)
200
150
100
50
0
0
0.05
0.1
0.25
0.5
1
Sodium metabisulphite (g/L)
C
Relative activity (%)
150
100
50
0
0
0.25
0.5
1
5
Malic acid (g/L)
D
Relative activity (%)
120
100
80
60
40
20
0
0
0.25
0.5
1
5
Tartaric acid (g/L)
E
Relative activity (%)
120
100
80
60
40
20
0
0
0.25
0.5
1
5
Lactic acid (g/L)
F
Relative activity (%)
140
120
100
80
60
40
20
0
0
0.25
0.5
1
5
Citric acid (g/L)
G
Relative activity (%)
120
100
80
60
40
20
0
0
2.5
5
10
15
20
25
NaCl concentration (%)