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Molecular Characterization of a Novel Arylesterase from the Wine-Associated Acetic Acid Bacterium *Gluconobacter oxidans* 621H

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ABSTRACT: An arylesterase from the wine-making acetic acid bacterium, *Gluconobacter oxidans*, was cloned and expressed into *Escherichia coli*. The soluble 76.8 kDa dimeric enzyme obtained, Est0881, was purified in only two steps with a 3.1-fold purification, 43% recovery, and a specific activity of 214 U/mg for the hydrolysis of *p*-nitrophenyl acetate. The optimum pH and temperature were 7.0 and 40 °C, respectively. The substrate specificity of this arylesterase was higher toward short chain *p*-nitrophenyl esters (C_2 to C_4) and also toward aromatic esters, such as phenyl acetate. The deduced amino acid sequence shares high identity with esterases of the HSL family. The inhibition results obtained showed that the enzyme was a serine esterase, belonging to the A-esterases (arylesterases) and contains a catalytic triad composed of Ser163, Asp263, and His293 in the active site. Est0881 retained significant activity under conditions simulating those of wine-making (75% activity at 20% ethanol), making it a promising biocatalyst for modulating the final aroma of wine.

KEYWORDS: *arylesterase, purification, Gluconobacter oxidans, wine aroma*

INTRODUCTION

The organoleptic properties of wine, such as flavor and aroma, depend on many factors, including grape variety, viticultural practices, the elaboration process, wine aging and storage conditions. During wine-making, the wide range of microorganisms that are present can release enzymes, which may influence wine aroma.[1] Among the compounds responsible for a winés aromatic profile, esters play a very important role. In wine, esters can be formed in two ways, enzymatically and by chemical esterification between alcohol and acids at the low pH values common during wine aging.[2] The esterases and lipases released into the wine come from the microflora, and are responsible for the synthesis of esters.[3−6]

Several authors have described the ester synthesis and hydrolysis activities of wine-associated yeast species[7] and lactic acid bacteria (LAB),[8] but only one esterase has been characterized from a LAB, *Oenococcus oeni*, which is used for wine-deacidification during malolactic fermentation.[8] However, little is known about the esterases from acetic acid bacteria (AAB), which are present at all stages of wine-making, from the mature grape, through vinification, to storage. A succession of *Gluconobacter oxidans*, *Acetobacter pasteurianus*, and *Acetobacter aceti* has been described during the course of these stages.[9] In fact, *G. oxidans* dominates the fresh must,[10] and is the predominant AAB after 7 dayś fermentation in red wines.[9] Among esterases, arylesterases are of biotechnological interest since they are not only active toward aliphatic esters but also toward aromatic ones (phenyl acetate), modulating the ester profile.[4,5]

In this paper, the molecular and enzymatic characteristics of a novel arylesterase from *G. oxidans* are reported, with a possible view to applying the esterase (in the conditions found in must and wine) in other industrial settings, such as cheese ripening, since the enzyme seems to function under harsh physicochemical conditions (extreme pH and alcohol concentration).

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Enzymes, and Reagents. *G. oxydans* 621H (CECT 360) was obtained from the Spanish culture collection (Valencia, Spain). *Escherichia coli* Rosetta (DE3) cells and pET28a plasmid were obtained from Novagen (Darmstadt, Germany). Restriction enzymes were obtained from New England Biolabs (Beverly, MA). T4 DNA ligase was from Roche Diagnostics (Barcelona, Spain), Pfu Turbo DNA polymerase and its 10× reaction buffer were from Stratagene (La Jolla, CA). The DNeasy system for genomic DNA isolation, QIA prep spin plasmid miniprep kit, and QIAquick PCR purification kit were from QIAgen (Hilden, Germany). Ultrafiltration, HisTrap chelating and PD10 columns were from GE Healthcare (Barcelona, Spain). All the substrates (*p*-nitrophenyl [NP] esters, of *S*-methyl thiobutanoate, phenyl acetate and naphthyl derivates), isopropyl-*β*-D-thiogalactopyranoside (IPTG), kanamycin, chloramphenicol, and standard proteins used in molecular mass studies were obtained from Sigma (Madrid, Spain). All other chemicals were of reagent grade and were obtained from commercial sources.

Cloning of Gene and DNA Manipulation. The whole genome of this strain was sequenced and published in GenBank (NCBI accession no. NC_006677), revealing that there is a putative gene (Gene ID 3250338), encoded by the putative arylesterase (YP191310.1).

General molecular biology techniques were used for cloning.[11] PCR amplification of *gox*0881 gene was carried out using *Pfu* Turbo DNA polymerase and the following oligonucleotides, which include the restriction sequence recognized by *Hin*dIII, Gox-up (5′-AAGCTTTGATGAACACACGCTCCCTCGTC-3′) and by *Xho*I, Gox-down (5′-GCTCGAGTCAGAGACCGAAGCCGCGTTT-3′). Primers were designed from the sequence of the putative arylesterase gene published in the NCBI database (Gene ID 3250338). This amplification yielded a product of 975 bp, which represents the entire *gox*0881 gene plus the sequence recognized by the restriction enzyme. The PCR product was

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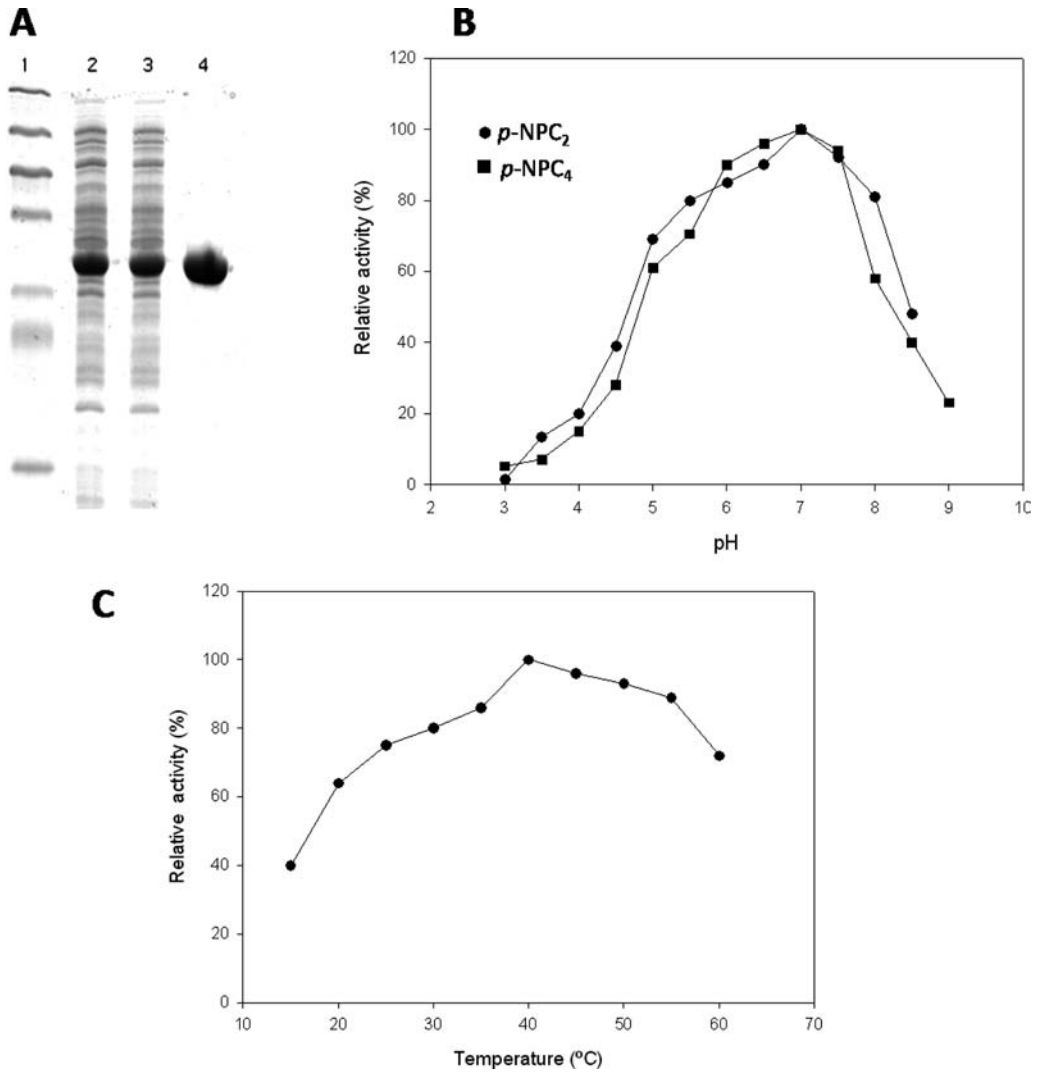

Figure 1. Biochemical characterization of Est0881. (A) SDS-PAGE of the Est0881 gene product after 8 h induction with 0.5 mM IPTG. Each lane contains 5 μg of protein. Lane 1, M: molecular weight markers (175 kDa β-galactosidase, 83 kDa myosine, 62 kDa glutamate dehydrogenase, 47.5 kDa aldolase, 32.5 kDa triosephosphate isomerase, 25 kDa β-lactoglobuline and 16.5 kDa lysozyme); lane 2, crude extract; lane 3, Ultrafiltration step; lane 4, His-Trap FF column fraction. (B) Effect of pH on Est0881 activity. The substrates used were *p*-NPC_2 and *p*-NPC_4. (C) Effect of temperature on Est0881 activity toward *p*-NPC_2. The esterase activity was measured in all cases in standard assay conditions, except for the tested parameter.

digested with *Hin*dIII and *Xho*I, purified by means of the QIAquick PCR purification kit, and inserted into the expression vector pET28a downstream of the T7 RNA polymerase promoter, which had been previously digested with the same restriction enzymes as the PCR product. This produced the recombinant plasmid pET28a6His-Est0881, which encodes for an additional 36 amino acid N-terminal tag, containing six histidines. Plasmids were transferred to *E. coli* Rosetta (DE3) electrocompetent cells and plated onto Luria-Bertaini (LB)-kanamycin-chloramphenicol agar plates. After overnight incubation, cells were picked and transferred into 20 mL shake-flasks containing LB-kanamycin-chloramphenicol supplemented with 0.5 mM IPTG. The correct sequence was checked by DNA sequencing.

Expression and Purification of Recombinant Est0881. Recombinant Rosetta (DE3) cells were grown at 37 °C in a Terrific Broth (TB) medium (1 L) containing 50 μg/mL kanamycin and 34 μg/mL chloranphenicol, and induced at different OD_{600} values, by adding IPTG at different concentrations to determine the optimum amount of inductor and different temperatures and times. The culture was centrifuged at 4 °C for 20 min at 6000*g*. The cell pellet was resuspended in 50 mM phosphate buffer pH 7.0 (80 mL), with protease inhibitor cocktail tablets (Roche, Barcelona, Spain). Resuspended cells were disrupted using a homogenizer mill (Mini ZetaII; Netzsch) for 15 min at 2000 rpm. The insoluble fraction of the lysate was removed by centrifugation at 4 °C for 20 min at 6000*g*. To remove nucleic acids, 3 U/mL DNase I (Sigma) was added, and the sample was shaken slowly for 30 min at room temperature, followed by centrifugation for 20 min at 6000*g* and 4 °C.

Enzyme purification was performed in two steps: 10-kDa tangential ultrafiltration on a QuixStand system (GE Healthcare), followed by affinity chromatography (ÄKTA Purifier, GE Healthcare) onto a HisTrap FF column (GE Healthcare). This column was equilibrated with 20 mM phosphate buffer, pH 7.0, containing 500 mM NaCl and 5 mM imidazol and the enzyme was eluted in a gradient from 5 to 500 mM imidazol at a flow rate of 1 mL/min. Fractions containing Est0881 were pooled, desalted onto a PD10 column, and stored at −80 °C.

Protein Analysis. The protein concentration was assayed by the bicinchoninic acid method,[12] using bovine serum albumin as a standard. The molecular weight of the native protein was determined by gel filtration (Superdex 200 10/300GL column; GE Healthcare) in 50 mM phosphate buffer, pH 8.0, 0.15 M NaCl, at a flow rate of 0.5 mL/min. The molecular mass under denaturing electrophoretic conditions (SDS-PAGE) was determined using 12% acrylamide gel.

Enzyme Assay. Esterase activity was determined spectrophotometrically through the hydrolysis of different nitrophenol esters (*p*-NP). The standard reaction medium (1 mL) contained 0.1 mM *p*-NP acetate, 100 mM phosphate buffer, pH 7.0, 10 μg/mL BSA and 0.25

Table 1. Purification of *G. oxydans* Est0881

step	volume (mL)	protein content (mg)	activity[a] (U)	specific activity (U/mg)	purification (*x*-fold)	yield (%)
Crude extract	250	1650	114450	69.3	1.0	100
Ultrafiltration	100	750	59500	79.3	1.1	52
His-trap FF	240	222	48884	214.3	3.1	43

[a]Enzyme activity was measured at 25 °C in 100 mM phosphate buffer pH 7 with *p*-NPC$_2$.

μg of purified enzyme at 25 °C. The degree of hydrolysis was measured at 405 nm (ε_{405} = 12 000 M^{-1} cm^{-1}) using a Shimadzu UV-2401PC spectrophotometer. One unit of activity was defined as the amount of enzyme releasing 1 μmol of *p*-NP/min under assay conditions.

Thioester activity was analyzed by the DTNB assay, in which the formation of free thiol groups from the thioester-catalyzed hydrolysis of *S*-methyl thiobutanoate was followed by monitoring the change of absorbance at 412 nm. The standard assay contained 100 mM Tris-HCl buffer, pH 8.0, 20 mM DTNB and different concentrations of *S*-methyl thiobutanoate. A stock solution of *S*-methyl thiobutanoate was prepared by dissolving substrate in acetonitrile. The absorption coefficient used for DTNB[13] was 13 700 M^{-1} cm^{-1}.

Naphthyl derivates were analyzed as described by Martínez-Martínez et al.[14] The arylesterase activity toward phenyl acetate was analyzed following the method described by Schulpis et al.[15]

Effect of Modulators. The effect of chemical modulators, such as denaturing agents, detergents, and organic solvents, was studied using the standard assay with *p*-NP acetate. The activity was measured at 25 °C, as described by Park et al.[16]

The inhibitory effect of several chemical modifiers which are specific for some amino acids of the enzyme, such as pyridoxal 5′-phosphate (PLP) for lysine, phenylglyoxal (PGO) for arginine, phenylmethylsulfonyl (PMSF) for serine, pestatin-A for aspartic acid, and diethylpyrocarbonate (DEPC) for histidine, was examined at two different concentrations (0.5 mM and 5 mM). The mixture was incubated for 30 min at 30 °C, and then the reaction was started by the addition of *p*-NP acetate. Paraoxon and Eserine, which are used for the classification of esterases, were also studied in the same conditions as described above.

Cations ($CaCl_2$, $MnCl_2$, $MgCl_2$, $AlCl_3$, NaCl, LiCl, $NiCl_2$, and KCl) at 5 mM were incubated with the enzyme at 30 °C for 30 min. To clarify whether the cations are required for the reaction, 10 mM EDTA was added to the purified enzyme, and then incubated for 30 min at 30 °C. The residual activities were measured using the standard assay with *p*-NP acetate.

Biochemical Characterization. The dependence of initial velocity on pH at 25 °C was monitored at 348 nm (the pH-independent isosbestic point of *p*-nitrophenol and the *p*-nitrophenoxide ion), using a molar absorption coefficient of 5300 M^{-1} cm^{-1}. The buffers used (100 mM) were citrate (pH 3.0−5.5), potasium phosphate (pH 6.0−7.5), Tris-HCl (pH 8.0) and glycine-NaOH (pH 8.5−11.0). Substrate specificity was assayed using different *p*-NP esters, such as *p*-NP acetate (C_2), *p*-NP butyrate (C_4), *p*-NP caprylate (C_8), and *p*-NP laurate (C_{12}). The optimal temperature was determined by *p*-NP acetate assay at temperatures from 20 to 60 °C. Finally, thermostability was determined using the standard assay with *p*-NP acetate, after incubation of the enzyme at the temperatures ranging from 40 to 60 °C for different periods of time.

Sequence and Structure Analysis. Protein similarity searchers and alignments were performed using the data from CLUSTALW,[17] and only sequences previously cloned and with high homology were used. The 3D structure was modeled with SWISS-MODEL, and rendered with Swiss-PdbViewer,[18−20] using heroin esterase from *Rhodococcus* sp. strain H1 (PDB code: 1LZL), as template. ESPript[21] was used to show multiple sequence alignments. Topology diagrams were prepared using the TOPS program.[22]

Table 2. Kinetic Parameter of Est0881 toward Different Substrates

substrate	K_M[a] (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (μM^{-1} s^{-1})
p-nitrophenyl acetate (C_2)	17.4 ± 0.1	185 ± 2	10.6
p-nitrophenyl butyrate (C_4)	174 ± 1	97.2 ± 0.1	0.56
Phenyl acetate	10 ± 0.1	231 ± 1	23.1
α-naphthyl acetate	7.9 ± 0.3	55.2 ± 0.3	6.9
β-naphthyl acetate	5.2 ± 0.1	99.5 ± 0.2	19.1
α-naphthyl butyrate	12.8 ± 0.1	7.0 ± 0.1	0.54
β- naphthyl butyrate	8.8 ± 0.2	16.4 ± 0.2	1.9
S-methyl thiobutanoate	7200 ± 10	20 ± 0.3	0.0028

[a]The activity was assayed in the standard reaction conditions, using different substrate concentrations. Results were mean ± SD from three independent experiments.

Table 3. Effect of Different Modulators and Organic Solvents on Est0881 Activity

	activity[a] (%)			
solvent	5% (v/v) solvent	10% (v/v) solvent	20% (v/v) solvent	40% (v/v) solvent
Ethanol	100	92 ± 0.2	75 ± 0.1	0
Methanol	100	90 ± 0.1	88 ± 0.1	57 ± 0.2
Acetonitrile	100	95 ± 0.1	56 ± 0.3	0
Acetone	100	93 ± 0.3	71 ± 0.2	0
DMSO	100	90 ± 0.1	74 ± 0.1	45 ± 0.1
Propanol	100	90 ± 0.2	58 ± 0.3	0
Inhibitor			0.5 mM	5 mM
Diethylpirocarbonate (DEPC)			0	0
Phenylglyoxal (PGO)			77 ± 0.3	60 ± 0.2
Phenylmethylsulfonyl fluoride (PMFS)			80 ± 0.1	80 ± 0.1
Pestatine A			86 ± 0.1	60 ± 0.1
Pyridoxal-5-phosphate (PLP)			62 ± 0.2	60 ± 0.3
Paraoxon (E600)			0	0
Eserine			42 ± 0.1	15 ± 0.1

[a]Reactions were carried out by pre-incubation of the enzyme at 30 °C in 100 mM phosphate buffer pH 7.0 for 30 min, with different concentrations of each modulator. Reactions were measured in standard reaction conditions using *p*-NPC$_2$. Results are mean ± SD from three independent experiments.

RESULTS AND DISCUSSION

Enzyme Cloning, Overexpression, and Purification. The *gox*0881 gene was amplified by PCR from genomic DNA extracted from *G. oxidans* 621H (CECT 360), using the oligonucleotide primers described in Materials and Methods. After purification, the PCR product was inserted into pET28a vector, and the recombinant plasmid pET28a6His-Est0881 was used to transform *E. coli* Rosetta (DE3) electrocompetent cells. The IPTG concentration and induction time were optimized (data not shown), with the highest specific activity being achieved after induction with 0.5 mM IPTG for 6 h at 30 °C. Est0881 was expressed in soluble form and represents, in these

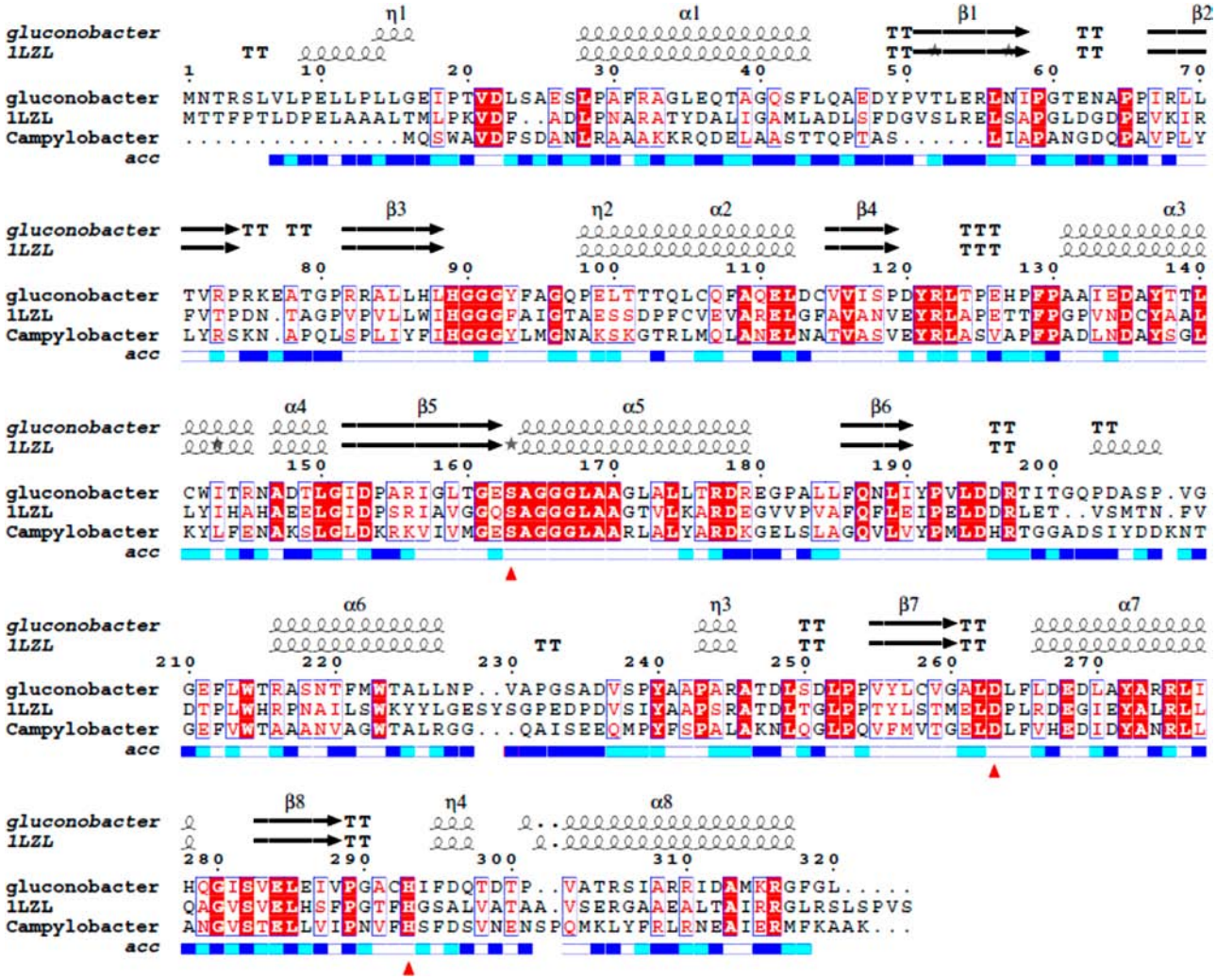

Figure 2. Multiple sequence aligment for Est0881 from *G. oxidans* 621H. Other esterases were a putative esterase from *Campylobacter* (NCBI code:), and an esterase from *Rhodococcus* sp. acetyl esterase, HerE (PDB code: 1LZL). Strictly conserved residues have a solid background. Symbols above blocks of sequences represent the secondary structure, springs represent helices, and arrows represent β-strands. The residues forming the hydrophobic specificity pocket are indicated by small black asterisks. Triangles represent the location of the active site.

conditions, about 34% of the total protein expressed, as calculated by image analysis with Image Quant TL software (Amersham Bioscences) (Figure 1A, lane 2).

The enzyme was purified from *E. coli* cells (1 L) grown in TB medium under the above conditions by a two-step procedure, consisting of a 10-kDa ultrafiltration step and Ni^{2+}-chelating affinity chromatography onto a HisTrap FF column. Following these steps, the enzyme could be considered pure, SDS-PAGE (Figure 1A, lane 4) pointing to a 3.1-fold purification and with a 43% recovery (Table 1). Est0881 showed a specific activity of 214 U/mg for the hydrolysis of *p*-NP acetate at 25 °C and pH 7.0 (Table 1). This specific activity was 10-fold higher than the only reported food-related arylesterase from *Lactobacillus casei LILA*.[5] Up to 222 mg of Ni^{2+} column purified enzyme could be obtained from 1 L of *E. coli* Rosetta (D3). This value was of the same order of magnitude as other industrial enzymes.[23] The molecular mass of the purified enzyme was determined by SDS-PAGE (35 kDa) (Figure 1A, lane 4), and by gel filtration chromatography (76.8 kDa), indicating the dimeric nature of Est0881.

Biochemical Characterization of Est0881. The activity of Est0881 was pH-dependent, being active over a broad pH range, from 5.0 to 8.0 with both substrates used (*p*-NPC_2 and *p*-NPC_4) (Figure 1B). The optimal pH of Est0881 was pH 7.0, which is similar to that of two esterases (esterase-1 and esterase-2) isolated from *A. pasteurianus*,[24] EstA of *Lactobacillus helveticus* CNRZ32[4,5] and EstB of *L. casei* LILA[5] but differs from that of *O. oeni* esterase,[8] whose optimum is pH 5.0. Interestingly, Est0881 maintained 5 to 20% activity under the pH conditions used in wine-making (pH 2.8 to 4.0). This residual level of activity at these pH values could be important for the modulation of ester profiles during fermentation, since these esters, which are potent flavoring compounds in the ppm range, act synergistically in wine, and do not need to be changed much to reach the desired level in a particular wine.[1]

The substrate specificity of purified Est0881 toward different acyl esters was determined (Table 2). Selectivity for *p*-NP esters was maximal for *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate, but decreased dramatically toward medium chain length esters, and no activity was detected toward long chain substrates, such as *p*-nitrophenyl caprylate (*p*-NPC_8) and *p*-nitrophenyl laureate (*p*-NPC_{12}) (data not shown). This substrate specificity was similar to that of wine-associated LAB *O. oeni* esterase,[8] but contrasts with that of EstB from *L.*

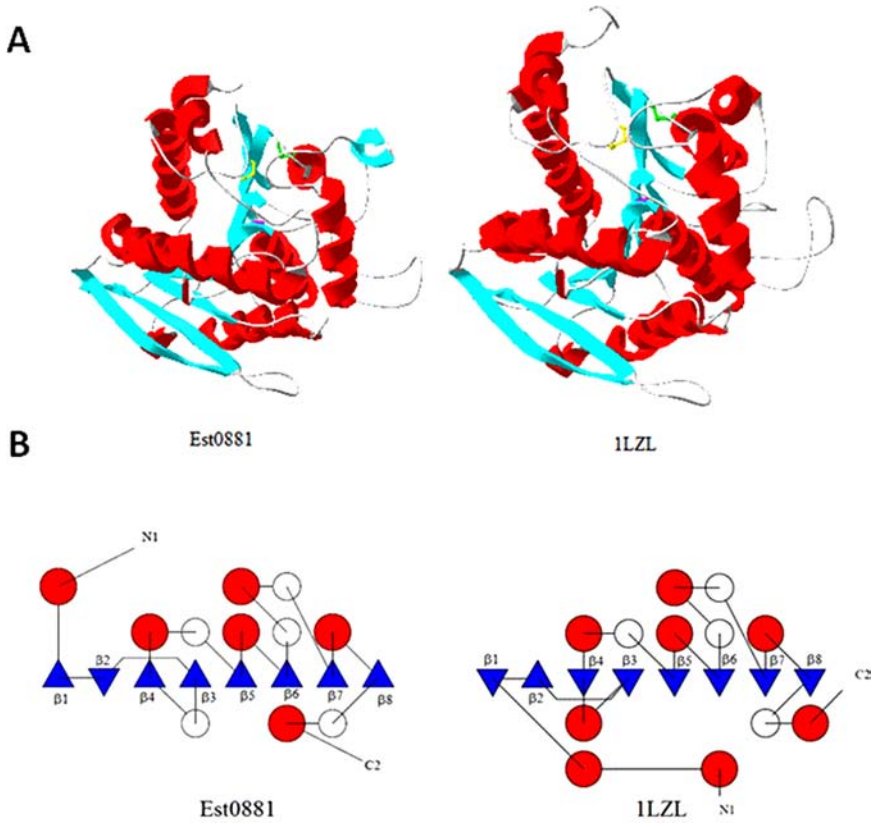

Figure 3. Structure and topology of Est0881. (A) Schematic representation of tertiary structure of Est0881 and 1LZL. Catalytic triads are shown in pink, green and yellow (Ser-Asp-His). β-Strands and α-helices belonging to the canonical α/β hydrolase fold are shown in blue and red, respectively. (B) Topology diagram of Est0881 and 1LZL. α-Helices are represented by circles and β-strands by triangles. White circles represent other small helices in the structure.

casei LILA,[5] whose selectivity toward ester substrates was greater for *p*-NPC_5 and *p*-NPC_6, suggesting that its binding pocket could accommodate longer alcohols than Est0881. In addition, Est0881 was also active toward phenyl acetate, indicating that the enzyme is an arylesterase (EC 3.1.1.2), similar to esterase-1 and esterase-2 from *A. pasteurianus*,[24] EstA from *L. helveticus*[4,5] and EstB from *L. casei* LILA.[5] Interestingly, Est0881 showed a similar catalytic efficiency toward aromatic esters (α-naphyl acetate and β-naphyl acetate) as toward *p*-NPC_2 (Table 2). Finally, Est0881 also showed moderate thiosterase activity toward *S*-methyl thiobutanoate, but its catalytic efficiency was 3−4 order of magnitude lower than toward the other esters assayed (Table 2).

The K_M values obtained increased as chain lengths increased (Table 2), confirming that the enzyme was more specific for short-chain fatty acids than for medium chain fatty acids, with K_M values in the range of micromolar (from 17 to 170 μM). These K_M values were similar to those obtained for wine-related LAB *O. oeni* esterase,[8] but contrast with the millimolar range values found in cheese-related *L. casei* EstB.[5]

Est0881 was affected by temperature over the range 40−60 °C, showing that it is not thermostable (data not shown). Maximal activity was found at 40 °C, using *p*-NPC_2 as substrate (Figure 1C). The profile obtained was compatible with wine-making practices (fermentation between 20 and 30 °C, and storage below 20 °C), since Est0881 retained 60% of its activity at 20 °C, and 80% of its activity at 30 °C.

Effect of Different Modulators. The effect of detergents and organic solvents on Est0881 was tested using the standard enzyme assay at 5% and 10% (v/v). SDS showed a denaturalizing effect over the enzyme at all the concentrations used. Triton X-100 produced a reduction of 25% in enzymatic activity, when used at a concentration of 5% (v/v), and a reduction of 47% at 10% (v/v). However, Tween 20 was an even more aggressive nonionic detergent, since only 16% and 7% of activity remained after the respective treatments (data not shown).

The addition of organic solvents, such as methanol, ethanol, acetonitrile, acetone, DMSO and propanol, had little effect on activity when used at 5% (v/v) (Table 3). However, when the solvents were used at 10%, a decrease in activity (5−10%) was observed. Interesting, Est0881 showed almost 75% activity at 20% ethanol. This is a remarkable feature for wine conditions, in which the ethanol concentration is about 14% (v/v). Such ethanol stability is a common feature of both wine-associated[8] and cheese-associated LAB arylesterases,[4,5] even though the ethanol levels are low during cheese ripening (about 6%).

The addition of divalent cations (data not shown) at 5 mM, and subsequent incubation for 30 min at 30 °C, did not affect enzymatic activity. These data, together with the fact that EDTA (10 mM) had no effect on activity, suggest that this esterase does not require a metal cofactor to express its activity, which is a typical feature of esterases.[25]

To identify the amino acids involved in the catalytic activity, the inhibition produced by several amino acid modifiers at 30 °C was tested at two concentrations (0.5−5 mM) after 30 min (Table 3). Est0881 was completely inhibited by diethylpyrocarbonate (DEPC) and paraoxon at 0.5 mM. The activity was

also significantly affected by phenylglyoxal (PGO), phenylmethylsulfonyl (PMFS), Pestatine-A, pyridoxal-5′-phosphate (PLP) and Eserine at the same concentration. These results suggest that serine, aspartic acid and histidine are involved in the activity of the enzyme. Phenylglyoxal (PGO) and pyridoxal also inhibited the activity, indicating that arginine and lysine also play an important role in Est0881 activity (Table 3).

Determination of Molecular Structure. *G. oxidans gox*0881 gene encodes a putative protein of 320 amino with a significant degree of identity with a putative esterase from *Campylobacter curvus* (NCBI accession no.) (42%), with *Rhodococcus* sp. acetyl esterase HerE (PDB code: 1LZL) (38%), with *Alicyclobacillus acidocaldarius* Est2 (PDB code: 1EVQ) (34%), with *Archeoglobus fulgidus* AFEST (PDB code: 1JJI) (32%) and with *Bacillus subtilis* brefeldin A (PDB code: 1JKM) (25%), all of which are classified in the HSL family.[25]

Multiple sequence alignment (Figure 2) revealed that Est0881 contains the typical catalytic triad composed of Ser163-Asp263-His293, and the consensus motif (Gly-X-Ser-X-Gly), where catalytic serine is located. These conserved residues are typical of α/β hydrolases, such as lipases and esterases. Apart from the above sequence, Est0881 also contains the His-Gly-Gly-Gly sequence (HGGG) located approximately 70 amino acids downstream of the catalytic serine, which is typical of enzymes belonging to the HSL family. This sequence is involved in the hydrogen bonding interactions that promote the stabilization of oxyanion hole and in the catalytic process.[26]

The three-dimensional structure of *Rhodococcus* sp. acetyl esterase, HerE (PDB code: 1LZL)[27] was used as a template to construct a model of the Est0881 (Figure 3). Est0881 has a single domain with a canonical α/β hydrolase fold, which typifies esterases with a Ser/Asp/His catalytic triad. The fold consists of a central, predominantly parallel (except β2), eight-stranded β-sheet (strand order 1-2-4-3-5-6-7-8) (Figure 3B), bordered by five main helices on one side and one on the other side. The β-α-β motifs are all left-handed, except for the unusual right-handed junction between strand β8 and the helix, which is needed to properly position the catalytic histidine residue in the active site (Figure 3A).[27]

The 3D structure also shows the presence of two separate helical regions external to the α/β core, forming a cap covering the active site. However, this putative cap appears to be structurally different from the lid occurring in various lipases,[28] since it allows direct access of catalytic serine to substrates and inhibitors, as has been described in recombinant human HSL,[29] using serine esterase inhibitors, such as phenylmethanesulfonyl fluoride (PMSF) and paraoxon (diethyl *p*-nitrophenyl phosphate or E600), in the absence of detergent. This inhibition was also found in Est0881, especially when using paraoxon, confirming the structural accessibility of catalytic serine.

In conclusion, Est0881 is the first esterase characterized from a wine-associated acetic acid bacterium, which retains activity under conditions relevant for wine-making. Further investigation is required to determine its physiological role and the role this enzyme plays in wine aroma.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

AAB, acetic acid bacteria; LAB, lactic acid bacteria; *p*-NPC$_2$, *p*-nitrophenyl acetate; *p*-NPC$_4$, *p*-nitrophenyl butyrate; IPTG, isopropyl-β-D-thiogalactopyranoside; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid

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