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Identification of a novel enzymatic activity from lactic acid bacteria able to degrade biogenic amines in wine

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Abstract The main objectives of this study were the search for enzymatic activities responsible for biogenic amine (BA) degradation in lactic acid bacteria (LAB) strains isolated from wine, their identification, and the evaluation of their applicability for reducing BAs in wine. Fifty-three percent of the 76 LAB cell extracts showed activity against a mixture of histamine, tyramine, and putrescine when analyzed in-gel. The quantification of the degrading ability for each individual amine was tested in a synthetic medium and wine. Most of the bacteria analyzed were able to degrade the three amines in both conditions. The highest percentages of degradation in wine were those of putrescine: up to 41 % diminution in 1 week. Enzymes responsible for amine degradation were isolated and purified from *Lactobacillus plantarum* J16 and *Pediococcus acidilactici* CECT 5930 strains and were identified as multicopper oxidases. This is the first report of an efficient BA reduction in wine by LAB. Furthermore, the identity of the enzymes involved has been revealed.

Keywords Biogenic amines · Histamine · Laccase · Lactic acid bacteria · Multicopper oxidase · Putrescine · Tyramine · Wine · *Lactobacillus plantarum* · *Pediococcus acidilactici* · *Trametes versicolor*

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Introduction

Biogenic amines (BAs) are low molecular weight, organic bases, frequently occurring in fermented food and beverages (Silla Santos 1996; Ten Brink et al. 1990). If they are present in high concentrations, these compounds can cause human health problems such as headaches, respiratory distress, heart palpitations, hyper- or hypotension, nausea, hot flush, sweating, bright red rash, and oral burning (Ten Brink et al. 1990). BAs can be present in raw food or be formed by microorganisms in fermented products, like wine. Twenty-four different BAs have been identified in wines, with putrescine being the most abundant and histamine the most dangerous (Lehtonen 1996; Vázquez-Lasa et al. 1998). In Europe, there are no regulations limiting the amount of histamine in wines, but the presence of this and other amines is considered a marker of poor quality and bad winemaking practices. In addition to the health concern, a high BA concentration in wine affects its organoleptical properties (Palacios et al. 2005). For these reasons, some European countries have recommended maximum levels for histamine, hampering the commercialization of wines that do not meet these recommendations.

Strategies that could be adopted to diminish BAs formation in wine are mainly (a) reducing precursor levels (amino acids), (b) limiting the growth of spoilage bacteria, and (c) inoculating starter cultures with no amino acid decarboxylases. When the aforementioned strategies fail or cannot be carried out, another strategy is to eliminate the BAs already produced through the use of microorganisms or enzymes. This strategy has been used in some food, but in the case of wine very little literature using this approach has been published. The ability to degrade biogenic amines in culture media or buffer systems by microorganisms has been reported in many papers. Some of these microorganisms were isolated from food. Thus, in 1978, Voigt and Eitenmiller mentioned the ability of some cheese lactic acid bacteria (LAB) to degrade

histamine and tyramine in culture media. Later, Leuschner et al. (1998) also found that some microorganisms isolated from food, LAB among them, degraded histamine and/or tyramine in buffer system. In 2000, Martuscelli et al. found that 21 out of 26 strains of *Staphylococcus xylosus* decreased histamine and/or tyramine in phosphate buffer (pH7). According to the results of Dapkevicius et al. (2000), some strains of *Lactobacillus sakei* and *Lactobacillus curvatus* isolated from mackerel fish paste were able to degrade histamine in MRS broth supplemented with this BA. In 2001, Fadda et al. described some *Lactobacillus casei* and *Lactobacillus plantarum* strains from meat possessing tyramine-degrading activities. Later, an extremely halophilic archaea *Natrinema gari* strain, isolated from anchovy fish sauce (Tapingkae et al. 2010), showed ability to degrade histamine in high-salt media. Recently, García-Ruiz et al. (2011) and Capozzi et al. (2012) reported that some wine LAB belonging to *Lactobacillus* and *Pediococcus* genera were able to degrade histamine, tyramine, and putrescine in culture media.

Some authors have used BA-degrading bacteria in order to diminish BAs in foods. Thus, Leuschner and Hammes (1998a) used three different strains of *Brevibacterium linens* as inocula to eliminate tyramine and histamine in cheese. Later, the same authors used one strain of *Micrococcus varians* in order to degrade tyramine during sausage ripening (Leuschner and Hammes 1998b). In 2000, Dapkevicius et al. used two strains of *L. sakei* for degrading histamine in fish slurry. In 2002, Gardini et al. managed to reduce concentrations of tyramine, spermine, and spermidine in dry sausages by inoculating a mixed starter of *L. sakei* G20 and *S. xylosus* S81. Later, in 2007, Yongsawatdigul et al. described that the inoculation of *Virgibacillus* sp. SK33 in Thai fish sauce fermentation halved the histamine content.

More recently, Mah and Wang (2009) reduced biogenic amine in Myeolchijeot, a salted and fermented anchovy (*Engraulis japonicas*), by using starter cultures of *S. xylosus* during ripening. Finally, García-Ruiz et al. (2011) attempted to reduce BAs in wine by using wine LAB but were unsuccessful.

In addition to microorganisms, amine oxidases (AOs) have been also used to reduce the content of BAs in foods. There are several patented procedures for obtaining enzymatic preparations of AOs from different sources, like animal organs and microorganisms, which could be used to degrade BAs in foods (Charles and Georgina 1985; Hiemenz and Setz 1942; Underberg and Lembke 1988; Williams 1943). In 1985, patent number EP0132674 described the preparation and use of an amino oxidase of *Aspergillus niger* IMI17454 to remove amines in cheese, beer, must, or yeast extract. However, although the authors mentioned that their procedure reduced BAs in grape must, they did not provide data on the efficiency of the method in must or in wine (Charles and Georgina 1985). Another patent reported the preparation of histaminases from bacterial and yeast cells of the genera *Lactobacillus* and *Candida* and their use to eliminate histamine from foodstuffs, beverages, and forages (Underberg and Lembke 1988). The use of a commercial diamine oxidase purified from porcine kidney is another option for histamine degradation. The activity of this enzyme was studied in phosphate buffer (pH7.0) and ensiled fish slurry (pH4.5) by Dapkevicius et al. (2000). Results reported no degradation at pH4.5, thereby limiting its use to food with higher pH values.

Microbial BA-degrading activities have been attributed to AOs. Thus, in 1965, one AO from *A. niger* was purified and characterized by Yamada et al (1965b). The first references of bacterial AOs were those of Yamada et al. (1965c) and Yamada et al. (1967) in which a putrescine oxidase of *Micrococcus rubens* (syn. *Kocuria rosea*) and a tyramine oxidase of *Sarcina lutea* (syn. *Micrococcus luteus*) were characterized. Later, Murooka et al. (1979) described that the enzyme responsible for BA oxidation in Gram-negative bacteria was a membrane-bound monoamine oxidase able to degrade tyramine, octopamine, dopamine, and norepinephrine. Afterwards, one methylamine oxidase was purified and characterized in 1981 from the yeast *Candida boidinii* (Haywood and Large 1981). Part of the above information was compiled and completed in the broad review by Yagodina et al. (2002) which also reported the existence of an AO in the archeobacterium *Methanosarcina barkery* and in the fungus *A. niger*. In 2004, a thermostable histamine oxidase was purified and characterized from the actinobacteria *Arthrobacter crystallopoietes* KAIT-B-007 (Sekiguchi et al. 2004).

No previous work has reported the successful removal of BAs from wine using purified and characterized enzymes or bacteria, even if the bacteria were of wine origin. The physicochemical conditions of wine, primarily low pH, prevent the development and activity of most bacteria, as well as the optimal performance of most described AOs.

So far as we know, the only work reporting successful degradation of BAs in wine is the study by Cueva et al. (2012). In this paper, the authors found an extracellular fraction of a vineyard *Penicillium citrinum* able to degrade histamine, tyramine, and putrescine in wine. However, many fungi, including some *P. citrinum* produce mycotoxins (Rundberget et al. 2004) or extracellular enzymes that can cause the browning of wine (Kassemeyer and Berkelmann-Löhnertz 2009). In contrast, LAB do not produce this kind of toxins and many of them have GRAS status; hence, their use in food fermentations is preferred. We aimed to detect wine LAB strains able to remove BAs of wines and identify the enzyme responsible for histamine, tyramine, and putrescine degradation. The use of LAB or their enzymes could solve the problem of high amine concentrations in wine, thereby improving the quality, safety, and competitiveness of wines in the market.

Materials and methods

Strains and growth conditions

Strains used belonged to our research ENOLAB collection, Spanish Type Culture Collection (CECT) and to the Reference Center for Lactobacilli (CERELA, Tucumán, Argentina). The majority of them were isolated from the winemaking process, but also reference strains from other habitats were included (Table 1). All strains were routinely grown overnight at 28 °C on modified MRS medium supplemented with L-cysteine 0.5 g/L and BAs (histamine, tyramine, and putrescine) at 10 mg/L each.

Obtaining of cell-free extracts and enzymatic assays

Cell-free extracts were obtained from cultures grown overnight in 50 mL of modified MRS (Scharlau) medium under conditions previously described. Cells were collected by centrifugation at 10,000 rpm for 10 min (Multifuge 1 S-R, Heraeus), washed twice with 25 mL of sodium phosphate buffer 50 mM pH7.4 and resuspended in 500 μL of the same buffer containing 1 mM of phenylmethylsulfonyl fluoride (PMSF) as protease inhibitor. Then, cells were disrupted with 1 g of 106 μM diameter glass beads in a Mikrodismenbrator® Sartorius: 10 cycles of 40 s, alternating 5 cycles of disruption with a cooling step of 5 min in ice. Whole-cell extracts were centrifuged at 13,000 rpm for 15 min (PrismR, Labnet), and supernatants were saved at −20 °C until use.

A volume of 25 μL of each cell-free extract was mixed with loading buffer containing 10 % glycerol, 50 mM Tris–HCl, pH6.8, bromophenol blue (0.02 % final concentration), loaded on a stacking 4 % polyacrylamide gel and resolved in a non-denaturing 8 % polyacrylamide gel, using Tris–glycine as electrophoretic buffer (25 mM Tris base, 192 mM glycine). Samples were electrophoresed for an hour at 30 mA. After protein separation, the gel was used for in-gel amine-degrading activity or multicopper oxidase (MCO) activity detection. In the first case, amine oxidase assay was performed following the procedure described by Leuschner et al. (1998), with some modifications: gel was placed in sodium phosphate buffer (50 mM, pH7.4) containing 1 mM of histamine, tyramine, and putrescine for 15 min, and then the solution was discarded and replaced by a new one of the same buffer containing horseradish peroxidase (1,000 U/L) and diaminobenzidine (DAB, 0.25 mM) as chromogenic substrate. The presence of amine-degrading activity was revealed by the apparition of a brown color on the active band after 1–2 h. Brown color was due to the precipitated product of DAB oxidation. In the case of MCO detection, after electrophoresis, gel was placed in sodium acetate buffer 100 mM at pH4 with 10 mM 2,6-dimethoxyphenol (DMP) for 5 min, and then the solution was discarded and replaced with a new one of the same buffer containing 1 mM $CuSO_4$. MCO activity was revealed after 10 min by the presence of an orange-yellow band.

Assays for quantifying amine degradation of cells in synthetic medium and wine

A volume of 100 μL of culture grown overnight on MRS supplemented with L-cysteine and BAs (as described above) was used to inoculate the medium described by Dapkevicius et al. (2000) with some modifications: 0.15 g/L of histamine, tyramine, and putrescine were added separately to the medium and the pH was adjusted to 5.5 (García-Ruiz et al. 2011). After 48-h incubation at 28 °C, the reaction was stopped by adding 1 M HCl. Then, samples were centrifuged at 13,500 rpm for 5 min and filtered through 0.22-μm nylon membranes (Fisher). Amine concentrations were measured by HPLC in an Agilent 1200SL HPLC system. The HPLC system was equipped with an in-line degasser, autosampler, column heater, and a fluorescence detector. Chromatographic separation was performed on HPLC Luna C18 silica Phenomenex column (250×4.6 mm) with a guard column (20×4.6 mm) of the same type. A solution of methanol was used as mobile phase A and a solution of 140 mM sodium acetate trihydrate and 17 mM TEA adjusted to pH5.05 as mobile phase B. Gradient conditions used for separation were described by Hernández-Orte et al.(2006). A sample volume of 10 μL was buffered with 25 μL of a solution containing 0.2 M sodium borate buffer (pH8.8) and 5 mM disodium EDTA. The derivatization reaction was performed by adding 15 μL of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC solution Waters) according to the optimized procedure described by Cohen and De Antonis (1994). Excitation and emission wavelengths of the fluorescence detector were set at 250 and 395 mm, respectively. A volume of 5 μL of the derivatized sample was injected into HPLC system. HPLC column temperature was kept at 65 °C. Flow rate was set at 2 mL/min. Total elution time was 70 min. Degradation ratio relative to the uninoculated medium was calculated after 48 h of incubation.

In order to quantify the degradation of BAs in wine, cells were grown in the semisynthetic must-wine medium number 7 (Pardo et al. 1992) to stationary phase, recovered by centrifugation, washed twice with 50 mM sodium phosphate buffer, pH7.4, and concentrated to obtain a cellular suspension of 10 $O.D_{600}$, which was placed in a red wine at pH3.5 supplemented with 40 mg/L of histamine, tyramine, and putrescine separately. After incubation with mild agitation at 28 °C for 7 days, the resulting reactions for the three amines were mixed in equal amounts, centrifuged, and filtered as described above. Amines from wine and medium samples were extracted with Oasis® MCX 1 cc extraction cartridges (Waters) as described by Peña-Gallego et al. (2009) and quantified by HPLC as described above.

Table 1 Lactic acid bacteria used in this work

LAB	Strains	Isolated from/culture collection
L. brevis	Lb 131, Lb 250	Red wine/ENOLAB
L. brevis	Lb 67	Grape must/ENOLAB
L. collinoides	Lb 373, Lb 404	Red wine/ENOLAB
L. casei	CECT 475^T	Cheese/CECT
L. curvatus	C9-19C [a], C13-48[a]	Sausages/Roig-Sagués
L. fermentum	CHMDW 5A	Red wine/ENOLAB
L. delbrueckii	CECT 286	Grain mash/CECT
L. hilgardii	L6, L21, L27, L41, L44, L56	Grape must/ENOLAB
L. farciminis	CRL 678[b]	Sausages/CERELA
L. mali	C46, Lb 44, 45, 47, 52, 53, 75, 110, 197, 206, 334	Red wine/ENOLAB
L. paracasei	L51 , Lb 54	Grape must/ENOLAB
L. paracasei	Lb 309, 340, 362, 365, 380, 444, 446L, 446R, 451	Wine/ENOLAB
L. plantarum	CECT 748^T	Pickled cabbage/CECT
L. plantarum	C24, C51,C145, J16, J33, J39, Lb 98, 132, 135, 140	Grape must/ENOLAB
L. plantarum	Lb 102, Lb 153, Lb 291	Wine/ENOLAB
L. plantarum	MRS 6, MRS 69A	Fermented pasta/ENOLAB
L. sakei	CECT 906^T	Sake/CECT
L. vini	CECT 7072^T	Red wine/CECT
L. vini	Lb 154, Lb 209P	Red wine/ENOLAB
P. acidilactici	CECT 5765^T	Barley/CECT
P. acidilactici	CECT 5930	Barley/CECT
P. parvulus	P205, P486BL, P487	Wine/ENOLAB
P. pentosaceus	MRS 12, 14, 44, 77	Fermented pasta/ENOLAB
E. faecium	C1	Wine/ENOLAB
E. faecium	C2	Fermented pasta/ENOLAB

[a]These strains were kindly supplied by Roig-Sagués

[b]This strain was received from CERELA as *L. casei* but it was renamed as *L. farciminis* after identification by 16S rDNA gene sequencing

Purification and identification of enzymes

Amine-degrading enzyme from Pediococcus acidilactici CECT 5930

The cell-free extract, obtained from 8 L of culture cells, was ultracentrifuged at 47,000 rpm for an hour in a Beckman L-70 Ultracentrifuge, with a SW55TI rotor and the resulting supernatant fractionated by ammonium sulfate precipitation. Solid $(NH_4)_2SO_4$ was added to obtain 50 % saturation, and after standing at 4 °C for 30 min, precipitated proteins were eliminated by centrifugation at 10,000 rpm for 30 min at 4 °C (Multifuge 1 S-R, Heraeus). The supernatant was brought to 75 % saturation, stirred for 30 min at 4 °C, and the precipitate, containing the majority of the BAs degrading activity as revealed in-gel assay, was collected by centrifuging as before. Precipitated proteins were then dissolved, dialyzed overnight against equilibration buffer (50 mM Tris–HCl, pH8, 0.01 M of NaCl, 0.05 % Tween 20), and loaded onto an anion exchange column (3.1×0. 75 cm) of Macro-Prep Q support (Bio-Rad), previously equilibrated in the same buffer. After washing, retained proteins were eluted with 20 mL of a linear gradient of NaCl concentration from 0.1 to 0.6 M in equilibration buffer. Fractions of 500 μL were collected and tested for protein content, by monitoring the A_{280} in a calibrated NanoDrop 2000c spectrophotometer (Desjardins et al. 2001), and for BA-degrading activity by in-gel staining with DAB. Fractions exhibiting the highest enzyme activity (more rapid color onset and more intense colored bands) were pooled and further purified by electrophoresis using a semipreparative native 8 % polyacrylamide gel and run for 1 h at 30 mA. After BA-degrading activity staining, brown bands from several identical lanes were cut, put together, fragmented into small pieces by passing them throughout a perforated Eppendorf tube, and resuspended in loading buffer (50 mM Tris–HCl, pH6.8, 10 % glycerol, 2 % sodium dodecyl sulfate (SDS), 1 % β-mercaptoethanol, and 0.02 % bromophenol blue). The suspension was loaded on an 8 % SDS-polyacrylamide gel which was run under similar conditions as described for native polyacrylamide gel electrophoresis (PAGE), but adding 0.1 % SDS to the electrophoresis buffer. The prestained SDS-PAGE MW Fermentas marker composed of 20 to 120 kDa blue-colored proteins was used. This second gel was also revealed by in-gel assay with DAB, and the resulting brown band was cut and sent for protein

identification to Central Support Service for Experimental Research of the University of Valencia (SCSIE). Quality of the purification was checked by Coomassie blue staining of a parallel lane loaded with the same sample. Tryptic digests of excised band gel were analyzed by matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS) on an Autoflex speed instrument (Bruker). Samples were prepared using 4-cyano-4-hydroxycinnamic acid as matrix on prespotted anchorchip targets (Bruker). Calibration was performed in an external mode using a peptide calibration standard kit (Bruker Daltonics). The spectra were processed using Flex Analysis 3.3 software (Bruker Daltonics). MS analysis peak lists were generated using the signals in the *m/z* 800 to 4,000 region, with a signal-to-noise threshold greater than 3. The SNAP algorithm included in the software was used to select the monoisotopic peaks from the isotopic distributions observed. After removing *m/z* values corresponding to usually observed matrix cluster ions, an internal statistical calibration was applied. Peaks corresponding to keratin and trypsin autolysis peptides were then removed. The resulting final peak list was used for identification of the proteins by peptide mass fingerprint. The most significant peptides were studied by MS/MS analysis. Mascot 2.2 program (Matrix Science Ltd, London UK) was used to search for homologies of these peptides in the NCBInr_20100430 database (13655082 sequences). Search parameters were trypsin cleavages excluding N-terminal to P, one or two missed cleavages allowed, cysteine carbamidomethylation set as fixed modification, methionine oxidation as variable modification, mass tolerance less than 50 ppm, and monoisotopic mass values. Criteria for positive identification were a significant Mascot probability score >84 ($p<0.05$).

Amine-degrading enzyme from L. plantarum ENOLAB J16

The procedure used was essentially similar to that described for *P. acidilactici* but using 10 L of culture and including an additional purification step necessary for final high-quality protein isolation. Seventy-five percent saturation ammonium sulfate precipitate was dissolved in equilibration buffer (50 mM sodium acetate, pH4.5, 5 mM NaCl, and 0.05 % Tween 20) and then dialyzed overnight at 4 °C against the same buffer. Dialyzed sample was centrifuged (13,500 rpm, 5 min, PrismR, Labnet) to eliminate the precipitate generated during dialysis, and the resulting supernatant was loaded onto a cation exchange column (3.1×0.75 cm) of sulfopropyl Sepharose FF (GE Healthcare) pre-equilibrated with the dialysis buffer. The column was then washed and retained proteins eluted with a linear gradient of NaCl concentration ranging from 5 to 500 mM, in the same buffer (total volume 20 mL). Fractions of 500 μL were collected and analyzed for protein content by spectrophotometry and for BA-degrading activity by in-gel assay. Fractions showing enzymatic activity were pooled, dialyzed against equilibration buffer (50 mM Tris–HCl, pH8.0, 0.1 M NaCl, 0.05 % Tween 20), and loaded onto an anion exchange chromatography on Macro-Prep Q, similarly as described above. After further purification by native and 10 % SDS-PAGE, the purified protein was finally sent to SCSIE for identification, following the procedure described above for *P. acidilactici* protein.

Amine degradation by the purified enzyme SufI and a commercial MCO (laccase) from *Trametes versicolor*

Purified protein from *L. plantarum* J16 was tested for amine degradation in a buffer model system containing 80 μL of 50 mM sodium phosphate buffer, pH6.5+150 mg/L of each BA separately. Twenty microliters of purified enzyme were added to the buffer and incubated at 37 °C for 48 h. A parallel assay adding the laccase mediator 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) at 10 mM was performed because most laccases are able to degrade some compounds only in the presence of a laccase mediator system. Laccase mediators can be natural or synthetic compounds that enhance enzyme activity by redox mechanisms, and ABTS is the most studied mediator (Morozova et al. 2007). Samples were subsequently analyzed by HPLC.

To test the ability of a known MCO to degrade amines, the activity of commercial *T. versicolor* laccase (Sigma) against BAs was tested by HPLC. Five units of this laccase were added to 2 mL of succinate buffer 0.2 M (pH4.5) plus histamine, tyramine, and putrescine, 1 g/L each final concentration and incubated at 37 °C for 24 h. As in the previous case, a parallel assay adding the laccase mediator ABTS at 5 mM was performed. In both cases, samples were taken at 0 and 24 h, and amine quantification was performed by HPLC analysis.

Primers design and amplification conditions for the detection of genes encoding the multicopper oxidases SufI and D2EK17

Specific PCR primers were designed and used for the detection of genes encoding MCOs from *L. plantarum* and *P. acidilactici*. From gene sequences encoding SufI and D2EK17 proteins, primers were designed for amplification of internal fragments of these genes in *L. plantarum* and *P. acidilactici* strains. The couple of primers Lac Lp1 (5′-CCCAGAATTGACGACTTTCC-3′) and Lac Lp2 (5′-GGATGGGATGGATGATGAAGT-3′) were designed to amplify an internal fragment of *sufI* gene of *L. plantarum*. Primers developed to amplify the gene encoding protein D2EK17 from *P. acidilactici* were Lac Pa1 (5′-CAAACAACTTGCCATCCAAC-3′) and Lac Pa2 (5′-GTCGGCTTTGTAATCTAGTTGA-3′). Fragment amplifications were performed in an Eppendorf thermocycler

programmed with the following thermal profile setup: initial denaturation (95 °C for 5 min), followed by 35 cycles of denaturation (94 °C for 1 min), primer annealing (55 °C for 1 min), and extension (72 °C for 1 min). Reactions were completed with 5-min elongation time at 72 °C followed by cooling to 10 °C. Template DNA was obtained from picked colonies dissolved in 10 μL of mili-U water. DNA from different strains belonging to the species *L. plantarum* and *P. acidilactici* were amplified using the primers and the amplification conditions described above, in order to detect the presence of the gene fragment encoding for MCO proteins. PCR products were resolved by electrophoresis in 1.2 % (*w*/*v*) SeaKem® LE agarose (FMC, Rockland, ME, USA) 0.5× TBE (45 mM Tris–HCl, pH8.0, 45 mM boric acid, and 1 mM EDTA) gels stained with ethidium bromide (0.5 μg/mL). Images were digitalized with a GelPrinter Plus from TDI (Madrid, Spain). The PCR product mass was evaluated with a DNA Ladder 1 KB plus™ High Range (Invitrogen). The partial nucleotide sequence of the amplified gene was determined by sequencing with the primers. The resulting sequences were submitted to the Basic Local Alignment Search Tool program available at the National Center for Biotechnology Information (NCBI, Bethesda, USA; http://www.ncbi.nlm.nih.gov) to search for similarities in the GenBank database. Mega5 alignment software was also used in the case of *P. acidilactici* sequences.

Results

Detection of amine-degrading activities in cell-free extracts by in-gel staining

Forty of the 76 extracts of LAB tested in-gel (52.6 %) showed a single brown band revealed with a mixture of histamine, tyramine, and putrescine in DAB assay. These results show that some LAB strains have enzymes able to degrade amines (Fig. 1a). Positive reactions were found in strains belonging to the species *Enterococcus faecium*, *Lactobacillus brevis*, *Lactobacillus collinoides*, *Lactobacillus delbrueckii*, *Lactobacillus farciminis*, *Lactobacillus hilgardii*, *Lactobacillus mali*, *Lactobacillus paracasei*, *Lactobacillus pentosus*, *L. plantarum*, *Lactobacillus vini*, *P. acidilactici*, *Pediococcus parvulus*, and *Pediococcus pentosaceus* (Table 2). Brown bands of different species or even strains appeared at different heights in the gel, suggesting that different enzymes or a number of subunits of the same enzyme could be responsible for amine-degrading activities (Fig. 1a). Table 2 shows that the species with the highest number of positive extracts was *L. plantarum*: all the extracts were positive, suggesting that the ability to degrade BAs could be a general trait for this species. *L. plantarum* and *P. acidilactici* strains exhibited higher intensity and quicker onset of brown color than the other extracts in-gel staining.

For the positive extracts of *L. plantarum* and *P. acidilactici*, additional tests in-gel were performed for each single BA. Results revealed that all of them degraded the three amines (results not shown). The fact that brown marks developed for each amine appeared at the same height in the gels leads us to infer that only one protein was responsible for the three activities.

BA degradation by cells in synthetic medium and wine

Thirteen LAB strains bearing amine-degrading enzymes were tested to quantify their histamine-, tyramine-, and putrescine-degrading ability in Dapkevicius' medium, as described above. Twelve strains were able to degrade histamine, four of them degraded up to 34 % (Table 3). Tyramine was degraded by eight strains, of which six of them reduced the initial concentration by a third. Putrescine was degraded by 12 strains but to a lesser extent than the other amines. Seven strains degraded the three amines (five of them belonged to *L. plantarum*, one to *L. delbrueckii*, and one to *P. acidilactici*).

Strains showing the highest degrading activity towards histamine were *L. plantarum* Lb 140 and *L. delbrueckii* CECT 286. Tyramine was better degraded by four strains of *L. plantarum*: Lb 132, Lb 98, Lb 291, and J16 (in decreasing order), *P. acidilactici* CECT 5930, and *L. farciminis* CRL 678 (positive control for tyramine degradation). The three best degraders for putrescine were two strains of *L. plantarum* (J16, Lb 291) and *L. farciminis* CRL 678 (Table 3).

The most degrading strains in Dapkevicius' medium were tested in wine. All *L. plantarum* and *P. acidilactici* CECT 5930 strains were able to degrade the three amines in wine although with different efficiencies. However, *L. farciminis* only degraded tyramine and putrescine but degraded the latter to a high degree (Table 4). In wine, degradation percentages reached 28 % for histamine (strain Lb 98), 28 % for tyramine (strain Lb 132), and more than 40 % for putrescine (strain Lb 98, *L. farciminis* CRL 678). Histamine was degraded in wine at a higher percentage than in synthetic medium by two strains of *L. plantarum* (J16 and Lb 98). The same phenomenon occurred in all cases for putrescine; however, tyramine degradation in wine was lower than in the medium.

Purification and identification of enzymes responsible for amine degradation in *P. acidilactici* CECT 5930 and *L. plantarum* J16

Amine-degrading enzyme from P. acidilactici CECT 5930

The BA-degrading activity present in whole-cell extract from *P. acidilactici* CECT 5930, revealed by in-gel assay

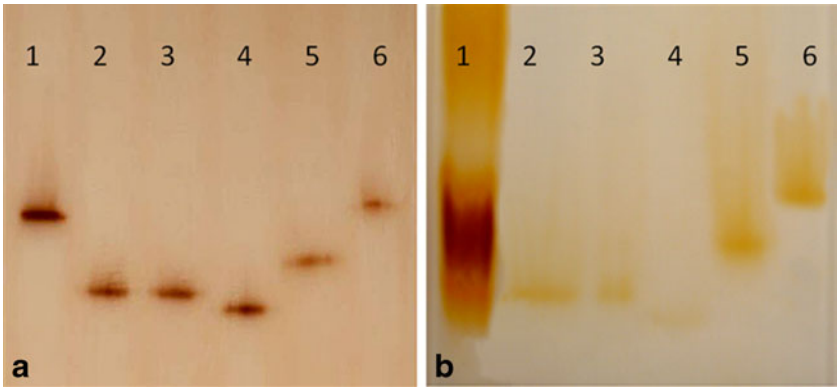

Fig. 1 **a** Amine-degrading activity showed by nondenaturing polyacrylamide gel staining from LAB cell-free extracts revealed with DAB. *Lane 1* is *T. versicolor* laccase, and *lanes 2 to 4* belong to *L. plantarum* J16, Lb 132, and Lb 291, respectively. *Lane 5* corresponds to *L. farciminis* CRL 678 and *lane 6* to *P. acidilactici* CECT 5930. **b** MCO activity by gel staining from LAB cell-free extracts and purified enzymes. *Lane 1* belongs to *T. versicolor* laccase, *lane 2* to purified protein SufI from *L. plantarum* J16, *lanes 3 and 4* belong to cell-free extracts from Lb 132 and Lb 291, respectively. *Lane 5* corresponds to *L. farciminis* CRL 678 extract and *lane 6* to the purified protein D2EK17 from *P. acidilactici* CECT 5930

using DAB, was purified by a combination of ammonium sulfate precipitation, anion exchange chromatography, and two different electrophoreses in polyacrylamide gels. Enzyme activity was recovered in the precipitate of 75 % saturation of ammonium sulfate fractionation, employed as the first step in the purification, and subsequently chromatographed on Macro-Prep Q. The BA-degrading activity eluted as a single peak around 300 mM NaCl, as revealed by in-gel with DAB assay. The fractions showing the highest levels of enzyme activity containing 11.5 mg/mL of protein were pooled and electrophoresed on a semipreparative native 8 % PAGE, followed by an SDS 8 % PAGE (strikingly, the enzyme retains its catalytic activity under SDS-denaturizing conditions, as shown in Fig. 2). The single brown band obtained after DAB gel staining was at the same position as a clear and isolated band visualized after Coomassie blue staining of a twin lane, indicating an excellent purification degree. Protein from the SDS-PAGE band was excised, digested with trypsin, and identified as a putative uncharacterized protein of *P. acidilactici* 7_4 with UniProt ID: D2EK17. This protein was cited in Prosite and Interpro Databases as a multicopper oxidase (http://www.ebi.ac.uk/interpro/IEntry?ac=IPR001117). Matched peptides covered the 36 % of the complete sequence of the identified protein (Fig. 3).

Amine-degrading enzyme from L. plantarum ENOLAB J16

Due to a lower enzymatic activity in *L. plantarum* ENOLAB J16 than in *P. acidilactici*, a larger amount of starting material was required and an additional purification step. A cation exchange chromatography on SP-Sepharose FF was added between ammonium sulfate precipitation and Macro-Prep Q chromatography. Dialysis steps with appropriated buffers were carried out in order to connect the different purification phases properly. Excellent purification was achieved when fraction containing 0.25 mg/ml of protein was loaded in native 8 % PAGE, followed by SDS-10 % PAGE (Fig. 4). The rest of the protein purification procedure and identification was essentially identical to that employed for *P. acidilactici* CECT 5930. Results from the Mascot Search software analysis showed that peptides (Fig. 5) pertained to the cell division protein SufI. This protein has been described as a provisional multicopper oxidase with accession number UniProt ID: C6VK53. Significant peptides covered 54 % of the complete protein sequence. In the laccase and multicopper oxidase engineering database (LccED) (http://www.lcced.uni-stuttgart.de), the cell division SufI was classified as MCO belonging to SUBFamily J (Bacterial CueO).

Evaluation of MCO activity from bacterial extracts and enzymes

To support the results provided by the Mascot identification software which identified purified proteins of *L. plantarum* and *P. acidilactici* as MCOs, their activity toward the canonical substrate of this kind of enzymes (DMP) was assayed in-gel (Fig. 1b). Yellow-orange bands appeared in-gel in both cases when DMP was added. The yellow-orange stained bands resulting after DMP staining were placed at the same positions as brown bands revealed with BAs and DAB in a twin-gel.

To find out if the enzymes responsible for amine degradation in the 76 cell-free extracts of LAB also had multicopper oxidase activity, they were tested in-gel with DMP as substrate. See Table 2 for the data. Forty-seven extracts (61 %) possessed activity toward DMP after 10 min of staining. Comparison of BA-degrading activity revealed with DAB shows that all the positive strains, except two, were also positive in

Table 2 Enzymatic activities of cell free extracts from LAB on biogenic amines (histamine, tyramine, and putrescine mixture) and DMP assayed under nondenaturing polyacrylamide gels

LAB strain	Enzymatic activities		LAB strain	Enzymatic activities	
	BAs	DMP		BAs	DMP
E. faecium C1	+	−	*L. paracasei* Lb 446L	+	+
E. faecium C2	+	−	*L. paracasei* Lb 446R	−	−
L. brevis Lb 67	+	+	*L. paracasei* Lb 451	−	+
L. brevis Lb 131	+	+	*L. pentosus* Lb 445	+	+
L. brevis Lb 250	−	−	*L. pentosus* Lb 453	+	+
L. casei CECT 475^T	−	−	*L. plantarum* CECT 748^T	+	+
L. collinoides Lb 373	+	+	*L. plantarum* C24	+	+
L. collinoides Lb 404	−	−	*L. plantarum* C51	+	+
L. curvatus C9-19C	−	−	*L. plantarum* C145	+	+
L. curvatus C13-48	−	−	*L. plantarum* J16	+	+
L. delbrueckii CECT 286	+	+	*L. plantarum* J33	+	+
L. farciminis CRL 678	+	+	*L. plantarum* J39	+	+
L. fermentum CHMDW 5A	−	−	*L. plantarum* Lb 98	+	+
L. hilgardii L6	−	+	*L. plantarum* Lb 102	+	+
L. hilgardii L21	−	−	*L. plantarum* Lb 132	+	+
L. hilgardii L27	−	+	*L. plantarum* Lb 135	+	+
L. hilgardii L41	+	+	*L. plantarum* Lb 140	+	+
L. hilgardii L44	+	+	*L. plantarum* Lb 153	+	+
L. hilgardii L56	−	+	*L. plantarum* Lb 291	+	+
L. mali C 46	−	+	*L. plantarum* MRS 6	+	+
L. mali Lb 44	−	−	*L. plantarum* MRS 69A	+	+
L. mali Lb 45	+	+	*L. sakei* CECT 906^T	−	−
L. mali Lb 47	−	−	*L. vini* CECT 7072^T	−	+
L. mali Lb 52	+	+	*L. vini* Lb 154	+	+
L. mali Lb 53	−	−	*L. vini* Lb 209P	−	−
L. mali Lb 75	+	+	*P. acidilactici* CECT 5765^T	+	+
L. mali Lb 110	+	+	*P. acidilactici* CECT 5930	+	+
L. mali Lb197	−	−	*P. parvulus* P 205	−	−
L. mali Lb 206	−	−	*P. parvulus* P 486 BL	−	−
L. mali Lb 334	+	+	*P. parvulus* P 487	−	+
L. paracasei L51	−	−	*P. parvulus* R210 1A	−	−
L. paracasei L54	−	+	*P. parvulus* R210 2B	−	−
L. paracasei Lb 309	−	−	*P. parvulus* R211A	+	+
L. paracasei Lb 340	+	+	*P. parvulus* R211B	−	−
L. paracasei Lb 362	−	−	*P. pentosaceus* MRS 12	−	−
L. paracasei Lb 365	−	−	*P. pentosaceus* MRS 14	−	−
L. paracasei Lb 380	−	−	*P. pentosaceus* MRS 45	+	+
L. paracasei Lb 444	−	+	*P. pentosaceus* MRS 77	+	+

the DMP assay. Twelve strains gave a positive response for DMP but not for DAB at the conditions used, and they belonged to *L. hilgardii*, *L. paracasei*, *L. vini*, and *P. parvulus* species. A special case is that of two strains of *E. faecium* that exhibited positive DAB staining in-gel but were not positives for DMP degradation in-gel conditions. This fact suggests that different enzymes may catalyze amine and DMP degradation. In this case, the enzyme able to degrade amines may be an AO.

The results confirm that the enzymes purified and identified used histamine, tyramine, putrescine, and DMP as substrates and are responsible for the oxidation of these compounds, although the chemical nature of the reaction is as yet unknown. In addition, the results compiled in Table 2

Table 3 Degradation percentages of three amines in modified Dapkevicius' medium supplemented with 150 mg/L of amines, and adjusted to 5.5 pH, after 48 h of incubation

LAB	Strain	Degradation (%)[a,b]		
		Histamine	Tyramine	Putrescine
L. delbrueckii	CECT 286	33±0.25	6.3±0.52	18.0±0.12
L. farciminis	CRL 678	n.e.	33.7±0.24	25.2±0.28
L. paracasei	ENOLAB Lb 444	11.3±0.12	n.e.	n.e.
L. plantarum	ENOLAB J16	4.7±0.17	33±0.13	26.2±0.42
L. plantarum	ENOLAB Lb 98	7.3±0.18	41.7±0.32	13.8±0.38
L. plantarum	ENOLAB Lb 132	15.3±0.55	42.9±0.22	14.5±0.15
L. plantarum	ENOLAB Lb 291	18.6±0.15	39±0.41	26±0.21
L. plantarum	ENOLAB J33	6.4±0.11	n.e.	14.8±0.58
L. plantarum	ENOLAB J39	16.4±0.21	n.e.	5.8±0.12
L. plantarum	ENOLAB Lb 140	33.9±0.25	8.6±0.46	15.7±0.51
L. plantarum	ENOLAB C145	14.6±0.32	n.e.	6.2±0.11
P. acidilactici	CECT 5930	13.8±0.15	40±0.23	19.3±0.14
E. faecium	C1	3.6±0.25	n.e.	16.8±0.13

n.e. no effect was observed

[a]Activity is expressed as a percentage of amine concentration present in the inoculated sample in relation to the uninoculated sample

[b]Mean values (n=3)

suggest that the same kind of MCO could be present in extracts showing positive reaction for BAs and DMP. A result supporting this hypothesis is the fact that the band stained brown (with amines and DAB) and yellow orange (with DMP) was located at the same position in twin gels (Fig. 1).

BA degradation by purified enzyme SufI and commercial laccase from *T. versicolor*

The percentages of degradation by purified SufI protein were 36 % for histamine, 80 % for tyramine, and 17 % for putrescine, in the presence of ABTS after 48-h incubation. In the absence of ABTS, degradations of histamine, tyramine, and putrescine were considerably lower, but tyramine was the least susceptible to the lack of mediator, with a 30 % of degradation.

To verify that known MCO could act on BAs, the commercial laccase of *T. versicolor* was assayed for amine degradation in the same way as the SufI protein. The assay was performed incubating the laccase with amines in the presence/absence of the mediator compound ABTS, and degradation quantifications were done by HPLC. After a 24-h incubation without ABTS, tyramine was degraded at almost 100 %, up to 42.2 % histamine was degraded but only in the presence of the mediator ABTS, and only 10 % of putrescine was eliminated in the presence or absence of the mediator.

The above results suggest that the enzyme responsible for degradation of amines in several LAB species is a single MCO enzyme acting on different substrates such as histamine, tyramine, putrescine, and DMP.

Amplification of genes encoding for SufI and D2EK17 proteins

The primers Lac Lp1/Lp2 were used to test for the presence of SufI encoding gene in the strains of *L. plantarum* described in Table 2. All strains tested showed a band of 765 bp, the expected size, and the sequencing results for *L. plantarum* J16 and *L. plantarum* CECT 748^T amplified fragments confirmed the presence of the gene encoding the protein SufI (Fig. 6). A nucleotide identity of more than

Table 4 Degradation percentages of three amines in red wine supplemented with 40 mg/L of amines

LAB	Strain	Degradation (%)[a,b]		
		Histamine	Tyramine	Putrescine
L. farciminis	CRL 678	n.e.	16.2±0.24	44±0.22
L. plantarum	ENOLAB J16	13.4±0.35	22.5±0.14	26.5±0.25
L. plantarum	ENOLAB Lb 98	27.8±0.21	25±0.11	41.1±0.34
L. plantarum	ENOLAB Lb 132	14.7±0.15	28.4±0.36	35.5±0.13
L. plantarum	ENOLAB Lb 291	15.6±0.16	17.8±0.52	29.8±0.22
P. acidilactici	CECT 5930	13.5±0.35	18.8±0.21	35.7±0.11

n.e. no effect was observed.

[a]Activity is expressed as a percentage of amine concentration present in the inoculated sample in relation to the uninoculated sample after one week incubation

[b]Mean values (n=3)

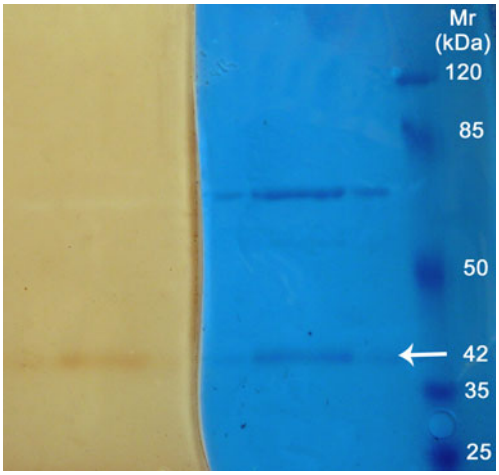

Fig. 2 8 % SDS-PAGE of purified protein from *P. acidilactici* CECT 5930. *Lane 1* purified protein stained with DAB, *lane 2* purified protein stained with Coomassie blue, *lane 3* molecular weight marker stained with Coomassie blue

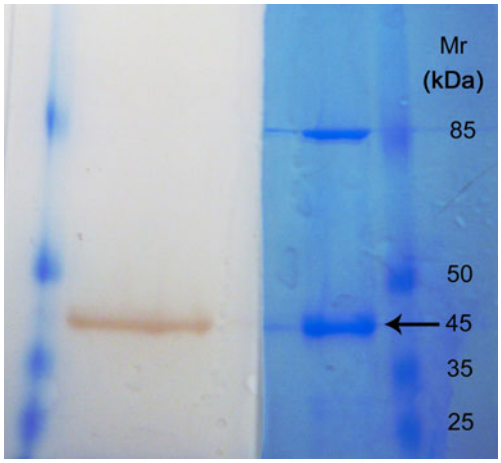

Fig. 4 10 % SDS-PAGE of purified protein from *L. plantarum* J16. *Lane 1* molecular weight marker, *lane 2* purified protein stained with DAB, *lane 3* purified protein stained with Coomassie blue, *lane 4* molecular weight marker stained with Coomassie blue

99 % with gene sequence GenBanK ID NC_012984.1 encoding SufI protein was recorded.

Amplification using the primers Lac Pa1/Pa2 and the conditions described in "Materials and methods" gave an expected fragment of 485 bp in the strains of *P. acidilactici* CECT 5765^T and CECT 5930 (Fig. 6). Sequences of both amplicons showed a sequence identity higher than 99 % with gene GenBanK ID NZ_GG730086.1 encoding for D2EK17 protein.

Thus, we can confirm the presence of the fragments from the genes encoding for MCOs in *L. plantarum* and *P. acidilactici* strains showing positive reaction for amine degradation and DMP oxidation.

Discussion

In this work, the screening of LAB able to degrade biogenic amines in wine was performed by a biochemical approach using the procedure used by Leuschner et al. 1998 to search for amine oxidase activities. The main reason for this methodology was to assure the presence of the enzymes catalyzing these reactions. The microbiological approach could give false-negative results if problems related to entry of the amine into the cell existed. The methodology used in this study has provided realistic information about the presence of enzymes acting on biogenic amines. From 76 LAB extracts screened, 40 of them (53 %) showed activity against BAs in-gel. Active extracts belonged to almost every species tested: *E. faecium*, *L. brevis*, *L. collinoides*, *L. delbrueckii*, *L. farciminis*, *L. hilgardii*, *L. mali*, *L. paracasei*, *L. pentosus*, *L. plantarum*, *L. vini*, *P. acidilactici*, *P. parvulus*, and *P. pentosaceus*. However, extracts from *L. casei*, *L. curvatus*, *L. fermentum*, and *L. sakei* gave negative results. In 1998, Leuschner et al. found lower percentages than us (42 %) of LAB able to degrade histamine or tyramine. The LAB species showing activity on histamine were *L. pentosus*, *L. plantarum*, *L. sakei*, and *P. acidilactici*, being one strain of *L. plantarum* and one of *P. acidilatici* the most active. Only one strain of *L. plantarum* was able to degrade tyramine. Differences in percentages of strains able to

Fig. 3 Mascot output corresponding to the identification of the protein of *P. acidilactici*. Peptides identified by MALDI-TOF in purified protein matching with several peptides of putative uncharacterized D2EK17 protein from *P. acidilactici* are indicated in *bold underlined letters*

MITKYL **YDENAYDYHDGGYRPLKKAPGEEHPLNVPAFLKPDR** IEGNEIYYTVTAQAGETK

ILPGKPTHTWGYNGSILGPAIQFETGK **TYHVTLK** NELDEVTTFHWHGLNIVGPYEDGGPH

APVYPHGERK**ITFTVDQPAANIWLHPHPCPETARQVWNGLAAPVIITDGHEQSLK**LPR**RW**

GVNDFPVVLQDRSYHDNQLDYK ADYDVDGTLGDYALVNGTVNPVVNVTKPIVRLRFLNGS

NRREWRLHFADYHPFTQIGSDGGLLPEAVEMDR **IMLTCAER** ADVLVNFSDYQPGQEVILQ

TDDFNLIKFKIGDIKKENMLLPSPLAEIPALSVDENTPVFK **TVMSGMDDQVRLDGKLFDMQR**

IDTRQQVDQTQIWEVSNTNDMEGGMIHPFHIHGCQFQLIDR **NGHAVNPNEHGWKDTIGVNPNETVR**

IKVKFTKLGIFMYHCHILEHEDTGMMAQIEIFDPDHPIEYHLMPMNHKM

MAKK **VYTDYFFDEPAYNTHDGGYIPLVTPKVEPQPLAIPPLLKPDRQTDTDDYYTVTAQE**

SETQFLPGKK TKTWGYNAGFLGQTIVFRNGK **QTHIDLENK** LPELTTFHWHGLNVPGPITD

GGCHAPVYPGETNHIDFKVHQPAATTWLHAHPCPSTATQVWK **GLATMVIIKDDVEDQLPL**

PRNYGVDDIPLVLQDREFHDDNQFDYRADYDPDGVQGHTALVNGTVNPYFDVTTQR VRLR

ILDGSNRR EWRLHFNDDLEFAQVASDGGILPAPVYMTK **VMMTCAER** DEIVVDFGQYQPGD

EVTLMTDDTPLCRFRIK **SFVPDDTKLPEHLVDIPDETPTPDLPVRTITMDGMDDEVALDG**

KKFDMSR IDAR **QKVGDVAIWEIRNTNSTENGMVHPFHVHGTQFR** VLAR **NDGPVYPNEHGL**

KDTVGVNPGETVR IKVKFELTGVYMYHCHIIEHEDGGMMAQIESYDPQHPQTYHLMDMDT

LRNAFAKEQGIKPEDVWMPGM

Fig. 5 Mascot output corresponding to the peptides identified in purified protein of *L. plantarum*. Matching of the peptides identified by MALDI-TOF with several peptides of *L. plantarum* SufI protein are indicated in *bold underlined letters*

degrade amines between the above study and our work could be attributed to the different approaches used in each work: microbiological versus biochemical. In both cases, various strains of *L. plantarum*, *P. acidilactici*, and *P. pentosus* showed positive responses for amine degradation. Our results show that the ability to degrade biogenic amines is strain dependent in the majority of the species, with the exception of *L. plantarum* in which all the extracts tested were active against a mixture of histamine, putrescine, and tyramine. This observation could mean that this activity is a general trait for this species and the results in Leuscher et al. (1998) support this affirmation. In some cases, the low number of extracts of different strains belonging to the same species is not enough to affirm that amine-degrading activity is strain dependent, as in the cases of *E. faecium*, *L. delbrueckii*, *L. farciminis*, *L. pentosus*, and *P. acidilactici*. Our results do not show a positive response of *L. sakei* CECT 906^T extract; however, Dapkevicius et al. (2000) reported strains of this species isolated from fish able to degrade histamine, thereby supporting the strain dependent activity in this species. However, the most active species in histamine and tyramine degradation belong to the actinobacteria group, especially *Arthrobacter*, *Micrococcus*, *Rhodococcus*, and *B. linens* strains, as Leuschner et al. (1998) and Fadda et al. (2001) have demonstrated. Nevertheless, these microorganisms are not described in the winemaking environment.

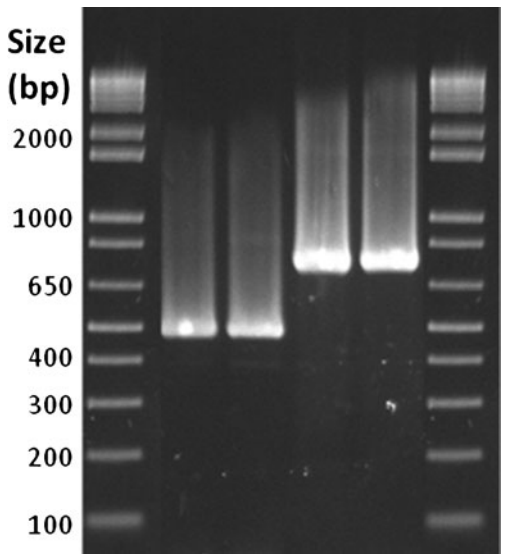

Fig. 6 Results of PCR amplification reactions obtained from *L. plantarum* and *P. acidilactici* strains. *Lanes 1 and 6* molecular weight marker 1 Kb Plus, *lanes 2 and 3 P. acidilactici* CECT 5930 and *P. acidilactici* CECT 5765^T amplification fragments obtained with Lac Pa 1/Pa 2, *lanes 4 and 5 L. plantarum* J16 and *L. plantarum* CECT 748^T amplification fragment obtained with Lac Lp1/Lp2

The degrading activity of wine strains whose extracts showed the quickest and strongest response in-gel was quantified for each individual amine. The most active wine strains degrading histamine, tyramine, and putrescine in Dapkevicius' medium belonged to *L. plantarum*, able to degrade up to 34, 43, and 26 %, respectively. Recently in 2012, Capozzi et al. reported five strains of *L. plantarum* able to degrade BAs. These results agree in part with the results published by García-Ruiz et al. (2011) in which the most active degrading strains of these three amines in culture medium were *Lactobacillus* and *Pediococcus* strains. However, Capozzi et al. (2012) did not test the activity of strains in wine and García-Ruiz et al., who did, found that none of the strains were active in wine. In our work, we have obtained percentages of degradation ranging from 13 to 30 % for histamine, 18 to 30 % for tyramine, and 26 to 41 % for putrescine, after 1 week of using *L. plantarum* strains. In some cases, the percentages of degradation were higher in wine than in the culture medium, pointing to the existence of mediator compounds in wine that collaborate in amine degradation. Dapkevicius et al. (2000) found a similar behavior in *L. sakei* strains: they degraded higher percentages of histamine in ensiled fish slurry than in synthetic medium.

The fact that brown bands appeared at the same position in-gels when they were revealed with each individual amine supports the idea that a single enzyme is responsible for the degradation of the three amines.

Until now, BA-degrading activities have been attributed exclusively to amine oxidases. Various authors have found bacteria able to degrade amines in several fermented foods (Dapkevicius et al. 2000; Fadda et al. 2001; García-Ruiz et al. 2011; Leuschner et al. 1998; Martuscelli et al. 2000; Tapingkae et al. 2010; Voigt and Eitenmiller 1978); and have even used them to reduce the amine concentration in cheeses, fermented sausages, fish, fish sauce, and fish slurry (Dapkevicius et al. 2000; Gardini et al. 2002; Leuschner and Hammes 1998a, b; Mah and Hwang 2009; Yongsawatdigul et al. 2007). All of them assumed that that the ability to degrade biogenic amines was due to the action of the AO; so therefore, the ability to degrade more than one amine would be due to the presence of several of these enzymes. However, none of them identified the enzymes responsible.

Several AOs have been identified in *Arthrobacter crystallopoietes*, *C. boidinii*, *Klebsiella aerogenes*, *M. rubens*, *S. lutea*, and *Rhodococcus erythropolis* (Cooper 1997; DeSa 1972; Haywood and Large 1981; Ishizuka et al. 1993; Murooka et al. 1979; Okamura et al. 1976; Ota et al. 2008; Sekiguchi et al. 2004; Van Hellemond et al. 2008; Yagodina et al. 2002; Yamada et al. 1965a, c), but never in LAB. Our results demonstrate that the ability to degrade BAs in at least two strains of *L. plantarum* and *P. acidilactici* is linked to the presence of a single enzyme. The purified enzyme from *L. plantarum* J16 showed two conserved domains present in protein SufI of *L. plantarum* and other MCOs compiled in the laccase and multicopper oxidase engineering database (LccED database). *P. acidilactici* protein D2EK17 is not included in this database, but it has the domain PRK10965 that is common to other MCOs; hence, it has been described as a putative MCO. We have proven the MCO activity of both enzymes, demonstrating their ability to oxidize DMP. In addition, we suggest that other strains belonging to these and other species have the same type of enzymes. The brown and yellow-orange bands appearing at the same position in twin gels when revealed with amines and DMP, respectively, supports the dual function of the enzyme. In addition, five of the species showing activity against BAs and DMP (*L. brevis*, *L. delbrueckii*, *L. paracasei*, *L. plantarum*, and *P. pentosaceus*) have enzymes classified in the same Subfamily J (Bacterial CueO) as the protein identified in *L. plantarum* J16 as can be seen in LccED database.

MCOs are encoded in the genomes of Eukarya, Bacteria, and Archaea. Their characteristic catalytic center contains four catalytic copper atoms conventionally classified into three types according to the copper's coordination and spectroscopic properties (Messerschmidt and Huber 1990). Type 1 (T1) copper (blue copper) is a mononuclear center involved in substrate oxidation, while T2 copper and binuclear T3 copper form a trinuclear cluster, the oxygen binding and reduction site.

With these four redox-active copper sites, the multicopper oxidases catalyze the four-electron ($4e^-$) reduction of dioxygen to $2H_2O$, an activity that they only share with terminal heme-containing oxidases. Most MCOs exhibit broad specificity toward various aromatic substrates such as diphenols, methoxy-substituted monophenols, and amines (Quintanar et al. 2007; Solomon et al. 1996). MCOs also can participate in cross-linking of monomers, degradation of polymers, and ring cleavage of aromatic compounds among other reactions (Sharma et al. 2007). The most studied MCOs are those belonging to lignin-degrading fungi, and these enzymes are mainly involved in wood decay. More recent are the descriptions of bacterial MCOs. Sharma et al. (2007) reviewed this kind of enzymes describing some of their characteristics, but the discovery of other novel bacterial MCO is in progress (Reiss et al. 2011).

Activity of MCO toward amines has been studied in very few works. In 2003, Arias et al. found a very low activity toward tyramine of a purified MCO from *Streptomyces cyaneus* CECT 3335. In 2008 and 2009, Kudanga et al., working on the functionalization of wood surfaces to bind fungicides, reported the ability of laccases to mediate the covalent binding of aromatic amines (including tyramine) to a lignin model molecule. They reported that tyramine was not a substrate for *Trametes hirsute* or *Bacillus* SF spore cotA laccases. In contrast, in this work we have demonstrated, for the first time, the ability of the *T. versicolor* laccase to degrade not only tyramine but also histamine and putrescine, although these latter two less efficiently.

As stated in the "Introduction," the only approach reporting a successful BA degradation in wine used a *P. citrinum* extracellular fraction (Cueva et al. 2012). The extract was not characterized by the authors, and they did not provide additional data about the nature of the enzyme responsible for BA degradation. As already stated, Rundberget et al. (2004) reported that some strains of the species *P. citrinum* are mycotoxin producers. The practical application to remove BA in wine with the Cuevas et al. procedure is not immediate. More research should be done to check for the presence of mycotoxins in the extract to ensure the safety of this procedure and to find out what enzyme is responsible for BA degradation. In our case, the *L. plantarum* strains able to remove BAs in wine have been isolated from the winemaking process and have been characterized as safe. In addition, the possibility of using purified MCO enzymes instead of microbial cells provides a new biotechnological alternative. Thus, our work offers the possibility of new safe procedures for reducing BAs in wine with biological approaches. However, more work on the enzymes is needed (biochemical and technological

characterization) before they can be used industrially. An exciting new research area awaits basic and applied results.

As far as we know, this is the first demonstration that LAB degrade BAs in wine and this is the first report in which the enzymes responsible for this reduction have been identified. They are MCOs and are able to degrade histamine, tyramine, and putrescine, in addition to their canonical substrate DMP. We have strong evidence that this kind of enzymes are present in various species of LAB and the most of them are active against the three amines, showing that these bacteria are a new source of MCOs. In addition, we have proven that laccase of *T. versicolor* is able to reduce tyramine, histamine, and putrescine.

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