Characterization of a benzyl alcohol dehydrogenase from *Lactobacillus plantarum* WCFS1

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Abstract

Aroma is an important sensory parameter of food products. Lactic acid bacteria have enzymatic activities that could be important in the modification of food aroma. The complete genome sequence from *Lactobacillus plantarum* WCFS1 shows a gene (lp_3054) putatively encoding a protein with benzyl alcohol dehydrogenase activity. In order to confirm its enzymatic activity we have overexpressed and purified lp_3054 from this strain. Protein alignment indicated that lp_3054 is a member of the family of NAD(P)-dependent long-chain zinc-dependent alcohol dehydrogenases. In lp_3054 all the residues involved in zinc and cofactor binding are conserved. It is also conserved the residue that determine the specificity of the dehydrogenase toward NAD+ rather than NADP+, and therefore, *L. plantarum* benzyl alcohol dehydrogenase is less active in the presence of NADP+ than in the presence of NAD+. The purified enzyme exhibits optimal activity at pH 5.0 and at 30 ºC. The kinetic parameters, K_m and V_{max} on benzyl alcohol as a substrate were, respectively, 0.23 mM and 204 μmol h^{-1} mg^{-1}. Besides its activity towards benzyl alcohol, it showed activity against nerol, geraniol, phenethyl alcohol, cinnamyl alcohol, and coniferyl alcohol, all of them volatile compounds involved in determining food aroma. The biochemical demonstration of a functional benzyl alcohol dehydrogenase activity in this lactic acid bacteria species should be considered when determining the influence of bacterial metabolism in the aroma of food products.

KEYWORDS: Benzyl alcohol dehydrogenase; benzyl alcohol; aromatic alcohols; aroma; benzaldehyde.

INTRODUCTION

Aroma is one of the most important quality criteria of fruit and wine products. Wine aroma, a very important sensory parameter, is composed of a wide variety of compounds with different aromatic properties. More than 800 volatile compounds have been identified in wine, including flavour compounds originating from the grape, yeast and bacterial fermentations, and post-fermentation treatments such as oak storage and bottle aging (*1*).

The typical aroma mainly relates to volatile compounds that come from the grapes. In a great number of fruits including grapes, apart from free flavour components, a significant part of important flavour compounds is accumulated as non-volatile and flavourless glycoconjugates, which are known as glycosidic aroma precursors. The odourless non-volatile glycosides, upon acid or enzymatic hydrolysis, can give rise to odorous volatiles or volatiles able to generate odour-active compounds during fruit juice processing or wine storage (*2*). Compounds such as terpenols, terpene diols, 2-phenylethanol, benzyl alcohol, and C_{13} norisoprenoids have been shown to be aglycons of such glycosides (*3*). Some of the monoterpenes alcohols are the most odoriferous, especially nerol and geraniol, which have floral aroma (*4*).

Lactobacillus plantarum is a lactic acid bacteria that is encountered in a variety of niches, being most abundant in the fermentation of plant-derived raw materials. *L. plantarum* strains are frequently used as malolactic starters in order to perform alcoholic and malolactic fermentations simultaneously when co-inoculated with selected yeast starter. Lactic acid bacteria associated with grape juice and wine has been shown to produce a range of enzymatic activities that could potentially be important in wine aroma. Several reports indicated that *L. plantarum* strains possess some glycosidase activities to hydrolyze

some odourless non-volatile glycosides to generate odour-active aglycons (*5*, *6, 7, 8*). However, the presence in *L. plantarum* of additional enzymatic activities able to modify the aglycons generated, such as some aromatic alcohols, remains unknown.

In year 2003, the complete genome sequence of *Lactobacillus plantarum* WCFS1 was available (*9*). From the analysis of this sequence, a protein annotated as aryl alcohol dehydrogenase (lp_3054) could be identified. Aryl alcohol dehydrogenase catalyzes the reversible oxidation of some aromatic alcohols to aldehydes with the concomitant reduction of NAD. Since i) aromatic alcohols are important volatile aroma compounds on foods; ii) aryl alcohol dehydrogenases has been scarcely reported in bacteria, and, only benzyl alcohol dehydrogenases from *Pseudomonas putida* (*10, 11*) and *Acinetobacter calcoaceticus* (*12, 13*) have been extensively characterized; iii) in fact no experimental evidence demonstrated that lp_3054 is an aryl alcohol dehydrogenase; we decided to genetically and biochemically characterize lp_3054 protein from *L. plantarum* WCFS1 in order to elucidate its role in the metabolism of this important food bacteria.

MATERIALS AND METHODS

Materials. The *L. plantarum* strain that has been complete sequenced, *L. plantarum* WCFS1 (NCIMB 8826), was used in this study. The compounds assayed in this study were benzyl alcohol (Fluka cat. no.13170), phenethyl alcohol (Fluka cat. no.77861), tyrosol (4-hydroxyphenethyl alcohol) (Fluka cat. no. 79058), coniferyl alcohol (Fluka cat. no. 27740), cinnamyl alcohol (Fluka cat. no. 96330), geraniol (Fluka cat. no. 48799), nerol (Fluka cat. no. 72170), ethanol (VWR BDH Prolabo), pyrogallol (Merck cat. no. 612),

catechol (Sigma cat. no. C9510), phloroglucinol (Fluka cat. no. 79330), tryptophol (Fluka cat. no. 54350), *p*-coumaric acid (Sigma cat. no. C9008), and caffeic acid (Sigma cat. no. C0625).

DNA manipulations. Bacterial DNA was isolated from overnight cultures using a protocol previously described (*14*). DNA sequencing was carried out by using an Abi Prism 377 DNA™ sequencer (Applied Biosystems, Inc.). Sequence similarity searches were carried out using Basic local alignment search tool (BLAST) on the EMBL/GenBank databases. Signatures, pI/MW, etc. were analysed on EXPASY (http//www.expasy.ch) site and multiple alignment was done using CLUSTAL W on EBI site (http://www.ebi.ac.uk) after retrieval of sequences from GenBank and Swiss-Prot.

Expression, and purification of benzyl alcohol dehydrogenase. Since the aryl alcohol dehydrogenases characterized in bacteria are generally designated as benzyl alcohol dehydrogenases (BADH), we decided to name the product of lp_3054 as benzyl alcohol dehydrogenase in order to unify designations. The gene coding for the putative benzyl alcohol dehydrogenase (lp_3054) from *L. plantarum* has been cloned and overexpressed following a strategy previously described (*15*). Briefly, the gene coding for lp_3054 from *L. plantarum* WCFS1 was PCR-amplified with Hot-start Turbo *Pfu* DNA polymerase by using the primers 323 (5´-*CATCATGGTGACGATGACGATAAG*atgaaaattaaagcagcagtggttg) and reverse 324 (5´-*AAGCTTAGTTAGCTATTATGCGTA*tttccccggttgataatcttcatc) (the nucleotides pairing the expression vector sequence are indicated in italics, and the nucleotides pairing the lp_3054 gene sequence are written in lowercase letters). The 1.1-kb purified PCR product was

inserted into pURI3 vector by using the restriction enzyme- and ligation-free cloning strategy described previously (*16*). Expression vector pURI3 was constructed based on the commercial expression vector pT7-7 (USB) but expressing a protein containing the following leader sequence MGGSHHHHHHGDDDDKM consisting of a N-terminal methionine followed by three spacer amino acids, a six-histidine affinity tag, a spacer glycine residue, and the five-amino acid enterokinase recognition site (*16*). *E. coli* DH5α cells were transformed, recombinant plasmids were isolated and those containing the correct insert were identified by restriction-enzyme analysis, verified by DNA sequencing and then transformed into *E. coli* JM109(DE3) (pLysS) cells.

Cells carrying the recombinant plasmid, pURI3-BADH, were grown at 37 ºC in Luria-Bertani media containing ampicillin (100 μg ml^{-1}) and chloramphenicol (34 μg ml^{-1}), and induced by adding 0.4 mM IPTG. After induction, the cells were grown at 22 ºC during 20 h and collected by centrifugation. Cells were resuspended in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl. Crude extracts were prepared by French Press lysis of cell suspensions (three cycles at 1100 p.s.i.). The insoluble fraction of the lysate was removed by centrifugation at 47 000 *g* for 30 min at 4 ºC (*17*).

The supernatant was filtered through a 0.45 μm filter and applied to a His-Trap™-FF crude chelating affinity column (Amersham Biosciences) equilibrated with 20 mM Tris-HCl, pH 8.0, 100 mM NaCl containing 10 mM imidazole, to improve the interaction specificity in the affinity chromatography step. The bound enzyme was eluted by applying a stepwise gradient of imidazole concentration, from 20 mM Tris-HCl, pH 8.0, 100 mM NaCl containing 10 mM imidazole to 20 mM Tris-HCl, pH 8.0, 100 mM NaCl containing 500 mM imidazole. Fractions containing the eluted BADH were pooled and the protein was then dialyzed overnight at 4 ºC in a membrane (3,500 cut-off) against 25 mM sodium

phosphate buffer, pH 6.5. The purity of the BADH enzyme was determined by 10% sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE) in Tris-glycine buffer.

Protein Assay. Protein concentration was measured according to the method of Bradford using a protein assay kit purchased from Bio-Rad Laboratories (Germany) with bovine serum albumin as standard.

Enzyme activity assay. Benzyl alcohol dehydrogenase (BADH) activity was assayed by following the rate of nicotinamide adenine dinucleotide (NAD+) reduction at 340 nm, since NAD+ is colourless at 340 nm whereas NADH absorbs strongly at this wavelenght (*10*). The NAD+ reduction reaction of BADH was measured in a reaction mixture (1.0 mL) containing 100 mM citric acid-sodium citrate buffer (pH 5.0), 15 mM ZnCl, 100 mM NAD+, and enzyme (3 µg), and the reaction was initiated with benzyl alcohol (final concentration 100 mM). The reduction rate at 30 ºC is linear between 5 and 40 min, and varies linearly with enzyme concentration. The standard assay was performed at 20 min, under the linear range of the enzyme reaction. Substrate and enzyme blanks were also prepared by incubating the enzyme or substrate with just buffer.

Enzyme characterization. The determination of kinetic parameters as well as the effect of different temperatures, pH, substrates, and additives on the activity of BADH from *L. plantarum* were studied. BADH activity was assayed by the standard assay described above. All the experiments were done in triplicate for each experiment and mean values calculated.

Kinetic analysis was performed at pH 5 and 30 ºC for 20 min in 100 mM citric acid-sodium citrate buffer containing the substrate (benzyl alcohol) at various concentrations. Values of K_M were calculated by fitting the initial rates as a function of substrate concentration to the Michaelis-Menten equation.

To determine the optimal pH of the BADH, the purified enzyme was incubated within different pH values (3-10) at 30 ºC for 20 min using benzyl alcohol (100 mM) as the substrate. Citric acid-sodium citrate buffer (100 mM) was used for pH 3-5, phosphate buffer (100 mM) for pH 6-7, Tris-HCl buffer (100 mM) for pH 7-8, and 100 mM glycine-KOH buffer for pH 9 and 10. The optimal temperature was assayed by incubating the purified BADH in 100 mM citric acid-sodium citrate buffer (pH 5.0) at different temperatures (4-90 ºC) for 20 min using 100 mM benzyl alcohol as substrate.

To determine the substrate specificity of the *L. plantarum* BADH different alcohols, allylic alcohols, aryl alcohols or phenolic compounds were assayed as potential substrates. These compounds were tested at 100 mM by the standard assay described above. The potential substrates assayed were phenethyl alcohol, tyrosol, conyferyl alcohol, cinnamyl alcohol, geraniol, nerol, ethanol, pyrogallol, catechol, phloroglucinol, tryptophol, *p*-coumaric acid, and caffeic acid.

Different metal ions (like Mg^{2+}, Mn^{2+}, Ni^{2+}, K^{+}, Na^{+}, Cd^{2+}, Ca^{2+}, and Hg^{2+}) surfactans (Tween 80 and SDS), denaturants (urea), chelators (EDTA), and inhibitors (DMSO, β-mercaptoethanol) were assayed at 10 mM final concentration. To assay the effect of metals and other additives on *L. plantarum* BADH activity, the enzyme was incubated with a 10 mM concentration of different additives and 100 mM benzyl alcohol 100 mM citric acid-sodium citrate buffer (pH 5.0) at 30 ºC for 20 min. The activity was calculated as relative activity to the sample containing no additives.

To validate the method the MINITAB Student test was used. Three replicate determinations were carried out for each experiment. Relative standard deviations were ≤ 5%.

RESULTS AND DISCUSSION

Benzyl alcohol dehydrogenase (lp_3054) from *L. plantarum* WCFS1 is a member of the family of NAD(P)-dependent long-chain zinc-dependent alcohol dehydrogenases. The deduced product of *L. plantarum* WCFS1 BADH is a protein of 373 amino acid residues, 40 kDa, and pI of 5.04. BLAST databases searches of the translated *L. plantarum* DNA sequence identified high-scoring similarities with the zinc-containing family of alcohol dehydrogenase protein sequences that catalyze the oxidation of ethanol to acetaldehyde with the concomitant reduction of NAD+. The predicted sequence of the *L. plantarum* WCFS1 BADH protein was aligned with selected zinc-containing alcohol dehydrogenases in the databases. The alignment of protein sequences that had the highest overall identity with that of *L. plantarum* is shown in **Figure 1**. The highest sequence identity (42-43%) was shown between *L. plantarum* and BADHs from a *Pseudomonas putida* plasmid and from *Acinetobacter* sp. strain ADP1. In addition, *L. plantarum* BADH showed a 31% identity to horse liver BADH (**Figure 1**).

Sequence alignment of members of the Zn-containing alcohol dehydrogenase family shows that only 9 amino acid residues are conserved in this family; these include eight Gly and one Val with structural roles (**Figure 1**) (*18*). Some of these residues constitutes the characteristic zinc-dependent alcohol dehydrogenase motif GHEXXGXXXXXGXXV

(from amino acid residues 61 to 76 in the *L. plantarum* BADH protein sequence) (*18, 19*). Crystallographic evidence has shown that horse-liver BADH, the archetypal enzyme of this family (**Figure 1**) binds two zinc atoms per enzyme subunit, with a catalytic zinc atom bound at the active site to the ligands Cys-46, Asp-49, His-67 and Cys-174, and a structural zinc atom bound to the cysteine residues 97, 100, 103, and 111 (**Figure 1**). These zinc ligands, except Cys-97, all are found to be conserved in the *L. plantarum* BADH protein (**Figure 1**). Several residues are involved in the cofactor binding in horse liver BADH. It has been described that Asp-223 is important in determining the specificity of the dehydrogenase toward NAD+ rather than NADP+. The residues Thr-178 and Val-203 interacts with the nicotinamide ring, polypeptide-backbone nitrogen atoms of residues Gly-201 and Gly-202 could interact with an oxygen of the phosphate proximal to the nicotinamide group, and Arg-47 binds the pyrophosphate of the NAD+ coenzyme. Position 47 in many enzymes are Arg, but substitutions to His, as in *L. plantarum* BADH protein, is frequently observed (*20*). As showed in **Figure 1**, except Gly-202, all the residues involved in NAD+ binding are conserved in the *L. plantarum* BADH. Sun and Plapp (1992) (*18*) described that the minimal requirements for dehydrogenases of this family include the following: several Gly residues at certain positions that are required to form a basic folded structure; residues that are necessary to bind the catalytic zinc and to modulate its electrostatic environment; and Asp residue that determine the specificity for NAD+; and finally, a Ser or Thr residue that facilitates proton removal from the substrate. Taking into account these requirements and based on its amino acid sequence, it could be concluded that lp_3054 from *L. plantarum* WCFS1 is a member of the family of NAD(P)-dependent long-chain zinc-dependent alcohol dehydrogenases.

Functional expression of lp_3054 from *L. plantarum* WCFS1. To confirm that the lp_3054 gene from *L. plantarum* WCFS1 encodes a functional BADH, we expressed this gene in *E. coli* under the control of the T7 RNA polymerase-inducible ϕ10 promoter.

Cell extracts were used to detect the presence of hyperproduced proteins by SDS-PAGE analysis. Control cells containing the pURI3 vector plasmid alone did not show expression over the 3-h time course analysed, whereas expression of additional 40-kDa protein was apparent with cells harbouring pURI3-BADH (**Figure 2A**). In addition, cells extracts from *E. coli* JM109(DE3) (pLysS) cells harbouring the recombinant plasmid pURI3-BADH were able to reduce NAD+, whereas extracts prepared from control cells containing the vector plasmid alone did not. Thus, we could prove experimentally that the lp_3054 gene encodes a functional BADH.

As the protein was cloned containing a purification poli-His tag, BADH was purified on a His-Trap™- FF crude chelating column and eluted with a stepwise gradient of imidazole. Highly purified BADH protein was obtained from pURI3-BADH (**Figure 2B**). The eluted BADH protein was dialysed to eliminate the imidazole, and checked for BADH activity. Spectrometric analysis demonstrated that highly purified BADH protein was able to reduce NAD+ (data not shown).

Enzymatic activity of *L. plantarum* BADH. In spite that benzyl alcohol dehydrogenases occur in a wide variety of organisms, including animals, plants, and yeasts (*18*), however, BADH have been scarcely found in bacteria, in fact they have been only biochemically demonstrated in some strains of the Gram-negative soil bacteria *Pseudomonas putida* and *Acinetobacter* sp. (*11, 12, 13, 21, 22, 23, 24*). So far this activity has never been described in bacteria involved in food processing.

As showed in **Figure 1**, *L. plantarum* BADH contains and Asp residue at the position equivalent to Asp-223 of the horse liver alcohol dehydrogenase, which has been shown to be important in determining the specificity of the dehydrogenase toward NAD+ rather than NADP+ (*24*). It has been determined that BADH is far less active in the presence of NADP+ than in the presence of NAD+. As expected, and by using benzyl alcohol as substrate, purified recombinant *L. plantarum* BADH showed one third lower activity using NADP+ than when using NAD+ as cofactor (data not shown).

As reported, the biochemical characterization of *L. plantarum* BADH was performed by using a standard assay with benzyl alcohol as substrate. **Figure 3A** shows that *L. plantarum* WCFS1 presented an optimal activity at 30 ºC, and remains quite stable from 30 to 50 ºC. At 70 ºC, BADH activity significantly decreased to 16% of residual activity. Temperature of 25 or 30 ºC have been often used to assay BADH activity in *Pseudomonas putida* (*25, 26*). The effect of pH on the enzyme activity was depicted in **Figure 3B** that shows an optimal pH around 5.0. However, this data is in contrast to the optimal pH for BADH activity in other bacteria, since optimal pH ranging from 9.2 to 9.5 have been reported for BADH from *Pseudomonas putida* or *Acinetobacter calcoaceticus* (*13, 25, 26*). This pH difference could be related to the low environmental pH generated by lactic acid bacteria growth.

Table 1 shows the results of *L. plantarum* BADH activity in presence of various additives added at 10 mM final concentration. BADH activity was neither activated nor inhibited when assayed in the presence of the following salts: KCl, NaCl, and $NiCl_2$. A similar behaviour was observed with these compounds at 1mM on BADH activity from *P. putida* (*20*); however, in this organism the activity of BADH nearly doubled when the concentrations of NaCl, KCl, and KBr increased from 0 to 25 mM, and this activity was

independent of the salt concentration when the concentration is between 75 and 400 mM (*20*). A lower increase on *L. plantarum* BADH activity (11%) was observed in the presence of 10 mM KBr (**Table 1**). The presence of $MgCl_2$, $CaCl_2$, and $CdCl_2$ slightly increased *L. plantarum* BADH activity (22-27%) (**Table 1**). It was also reported that *P. putida* BADH activity was increased (30-40%) by the presence of 1-10 mM $MgCl_2$ and, that BADH was stabilized by 2-mercaptoethanol and dithiothreitol, although concentrations of dithiothreitol higher than 2.5 mM were inhibitory (*26*). BADH from *L. plantarum* was strongly inhibited by 10 mM concentration of 2-mercaptoethanol and dithiothreitol (**Table 1**). The 2-mercaptoethanol inhibition was expected since the *L. plantarum* BADH protein contains several cysteine residues. The *P. putida* enzyme was not affected by the presence of 1-10 mM EDTA (*13*, *26*); however, *L. plantarum* BADH was partially inhibited by 10 mM EDTA (**Table 1**). Similarly to horse liver and yeast alcohol dehydrogenases, *L. plantarum* BADH enzyme is sensitive to chelating agents and this is known to be a result of the presence of zinc atoms at the active sites (*13*).

Substrate specificity of *L. plantarum* BADH. The kinetic parameters of the enzyme for benzyl alcohol were determined. The Lineweaver-Burk plot indicated that the K_m and V_{max} were, respectively, 0.23 mM and 204 μmol h^{-1} mg^{-1} (data not shown). K_m values ranging from 0.017 to 0.233 mM for benzyl alcohol were previously reported for BADH from *A. calcoaceticus* (*12*) and from *P. putida* (*27*).

Horse liver alcohol dehydrogenase has exceptionally broad substrate specificity and will oxidize both aliphatic and aromatic alcohols; benzyl alcohol is oxidized at approximately the same rate as ethanol (*28*). However the alcohol specificity of *A. calcoaceticus* BADH resembles that of some other bacterial aromatic alcohol dehydrogenases. They are in general specific for aromatic alcohols, with a preference for

aromatic alcohols with small substituents groups (*13*). We tested several compounds frequent in food substrates (**Table 2**). As expected, non-alcohol compounds (e.g. *p*-coumaric acid and caffeic acid) as well as some non-aromatic alcohols (e.g., ethanol) were not reduced by BADH. From the compounds assayed, benzyl alcohol was an effective alcohol substrate for *L. plantarum* BADH. The other substrates of BADH were most of them aromatic in nature. It has been described that the electron-withdrawing properties of the substituent groups on the aromatic ring may also be involved in dictating the acceptability of a particular substrate (*13*). BADH is in general specific for aromatic alcohols, or cyclic alcohols such as perillyl, cinnamyl and coniferyl alcohol; however, some BADH catalyze efficient oxidation of some allylic alcohols, such as geraniol and nerol (*29*). Our results indicated that BADH from *L. plantarum* was able to oxidize efficiently both terpenols, geraniol and nerol, which are important components of the wine aromatic fraction. In wine, García-Moruno et al. (2002) (*30*) studied the metabolism towards geraniol by wine *Saccharomyces cerevisiae* strains but not by lactic acid bacteria. Their results proved that the yeast strain dramatically affects the amount of geraniol occurring in the medium at the end of fermentation. They concluded that in the fermentation of aromatic musts in which geraniol is the main component, the choice of the yeast proves decisive for the quality of wine.

As described previously for *A. calcoaceticus* and *P. putida*, coniferyl alcohol and cinnamyl alcohol were also substrates (*13, 31*) (**Table 2**). Both aromatic alcohols have an alkenyl group between the reactive carbinol and the aromatic ring. Cinnamyl alcohol and coniferyl alcohol are both known intermediates of lignin biosynthesis and degradation. Although cinnamyl alcohol or, presumably, coniferyl alcohol cannot serve as sole carbon source for *L. plantarum*, their oxidation could give some energy to the bacteria and the

compounds produced would then be available for metabolism by other microorganisms. It may well be that in natural food substrates the mixed populations of microorganisms contain individual species that can metabolize compounds only partially, excreting or releasing those compounds that are then available to be utilized by other organism. It has been postulated that it is presumably by this route that *A. calcoaceticus* and other soil bacteria encounter aromatic alcohols, aldehydes and acids as a result of fungal degradation of lignins (*13*).

In the previously characterized BADHs, aromatic alcohols usually found in food substrates were not analysed, therefore we assayed several of these alcohols such as tyrosol, tryptophol, pyrogallol, catechol, or phloroglucinol as potential DADH substrates (**Table 2**). None of these alcohols were oxidize by BADH. These results are in agreement with those obtained when the *L. plantarum* metabolism of some phenolic compounds was studied, as tyrosol, frequent in olive products, or tryptophol, frequent in wine, were not modified by *L. plantarum* cultures or cell-extracts (*32*, *33*). In addition, it has been described that some phenolic acids were decarboxylated by *L. plantarum* strains giving their corresponding alcohols, like catechol from protocatechuic acid, and pyrogallol from gallic acid; nevertheless, these alcohols were nor further modified (*32, 34*). Now, in this work we have demonstrated that catechol and pyrogallol were neither oxidized by BADH in *L. plantarum*.

Among the aromatic alcohols oxidized by *L. plantarum* BADH, benzyl alcohol and phenethyl alcohol, as well as benzaldehyde produced, are volatile compounds which respective concentration vary considerably among grape varieties. Concentrations of benzyl alcohol up to 89.57 µg/L, phenethyl alcohol from 43.45 to 1635.38 µg/L, and 2.53 to 13.19 µg/L of benzaldehyde has been described in some *Vitis vinifera* grape varieties (*35*). These aromatic compounds, together with hexyl acetate, have been proposed to be used as a basis

for must and wine varietal differentiation. A similar study allowed benzyl alcohol to be selected as predictor variable in a discriminating analysis. This analysis indicated that benzyl alcohol depends on grape variety, skin maceration, or both (*36*). However, in this work we have demonstrated that *L. plantarum* strains possess a BADH enzyme able to modify benzyl alcohol levels in wines. Therefore in the search for compounds enabling the chemical differentiation of must and wines from grapes of different varieties, it should be take into account that some lactic acid bacteria species posses BADH enzymatic activities that could modify the levels of some of these aromatic alcohols.

In conclusion, lp_3054 protein from *L. plantarum* WCFS1 showed BADH activity able to oxidize some compounds involved in the aroma of food products, such as grape must or wine. Nerol, geraniol, benzyl alcohol, phenethyl alcohol, cinnamyl alcohol, or coniferyl alcohol could be oxidized by *L. plantarum* BADH. The presence of this enzymatic activity on *L. plantarum* strains will modify the aroma of food substrates, e.g. wines. In wine bacterial BADH activity should be considered in order to establish the aromatic alcohols selected as predictor variables for grape varietal differentiation.

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LITERATURE CITED

(1) Ebeler, S. E. Analytical chemistry: unlocking the secrets of wine flavor. *Food Rev. Int*. **2001**, 17, 45-64.

(2) Pogorzelski, E.; Wilkowska, A. Flavour enhancement through the enzymatic hydrolysis of glycosidic aroma precursors in juices and wine beverages: a review. *Flavour Frag. J*. **2007**, 22, 251-254.

(3) Genovés, S.; Gil, J. V.; Vallés, S.; Casas, J. A.; Manzanares, P. Assessment of the aromatic potential of Palomino Fino grape must using glycosidases. *Am. J. Enol. Vitic*. **2005**, 56, 188-190.

(4) García-Moruno, E. The chirality of α-terpineol in aromatic wines. Detection of chiral or racemic linaool addition in wines. *Sci. Alim*. **1999**, 19, 207-214.

(5) Marsilio, V.; Lanza, B.; Pozzi, N. Progress in table olive debittering: degradation in vitro of oleuropein and its derivatives by *Lactobacillus plantarum*. *J. Am. Oil Chem. Soc.* **1996**, 73, 593-598.

(6) Sestelo, A. B.; Poza, M.; Villa, T. G. Beta-glucosidase activity in a *Lactobacillus plantarum* wine strain. *World J. Microbiol. Biotech.* **2004**, 20, 633-637.

(7) Grimaldi, A.; Bartowsky, E.; Jiranek, V. Screening of *Lactobacillus* spp. and *Pediococcus* spp. for glycosidase activities that are important in oenology. J. Appl. Microbiol. **2005**, 99, 1061-1069.

(8) Spano, G.; Rinaldi, A.; Ugliano, M.; Moio, L; Beneduce, L.; Massa, S. A β-glucosidase gene isolated from wine *Lactobacillus plantarum* is regulated by abiotic stresses. *J. Appl. Microbiol*. **2005**, 98, 855-861.

(9) Kleerebezem, M.; Boekhorst, J.; van Kranenburg, R.; Molenaar, D.; Kuipers, O. P.; Leer, R.; Tarchini, R.; Peters, S. A.; Sandbrink, H. M.; Fiers, M. W. E. J.; Stiekema, W.; Klein Lankhorst, R. M.; Bron, P. A.; Hoffer, S. M.; Nierop Groot, M. N.; Kerkhoven, R.; de Vries, M.; Ursing, B.; de Vos, W. M.; Siezen, R. J. Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proc. Natl. Acad. Sci. USA*. **2003**, 100, 1990-1995.

(10) Worsey, M. J.; Williams, P. A. Metabolism of toluene and xylenes by *Pseudomonas putida* (*arvilla*) mt-2: Evidence for a new function of the TOL plasmid. *J. Bacteriol*. **1975**, 124, 7-13.

(11) Inouye, S.; Nakazawa, A.; Nakazawa, T. Molecular cloning of TOL genes *xylB* and *xylE* in *Escherichia coli*. *J. Bacteriol*. **1981**, 145, 1137-1143.

(12) MacKintosh, R. W.; Fewson, C. A. Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II from *Acinetobacter calcoaceticus*. Purification and preliminary characterization. *Biochem. J*. **1988**, 250, 743-751.

(13) MacKintosh, R. W.; Fewson, C. A. Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II from *Acinetobacter calcoaceticus*. Substrate specificities and inhibition studies. *Biochem. J*. **1988**, 255, 653-661.

(14) Vaquero, I.; Marcobal, A.; Muñoz, R. Tannase activity by lactic acid bacteria isolated from grape must and wine. *Int. J. Food Microbiol*. **2004**, 96, 199-204.

(15) Rodríguez, H.; de las Rivas, B.; Muñoz, R.; Mancheño, J. M. Overexpression, purification, crystallization and preliminary structural studies of *p*-coumaric acid decarboxylase from *Lactobacillus plantarum*. *Acta Cryst*. **2007**, F63, 300-303.

(16) De las Rivas, B.; Curiel, J. A.; Mancheño, J. M.; Muñoz, R. Expression vectors for enzyme restriction- and ligation-independent cloning for producing recombinant His-fusion proteins. *Biotech. Progress* **2007**, 23, 680-686.

(17) Muñoz, R.; López, R.; de Frutos, M.; García, E. First molecular characterization of a uridine diphosphate galacturonate 4-epimerase: an enzyme required for capsular biosynthesis in *Streptococcus pneumoniae* type 1. *Mol. Microbiol.* **1999**, 31, 703-713.

(18) Sun, H. W.; Plapp, B. V. Progressive sequence alignment and molecular evolution of the Zn-containing alcohol dehydrogenase family. *J. Mol. Evol.* **1992**, 34, 522-535.

(19) Gillooly, D. J.; Robertson, A. G. S.; Fewson, C. A. Molecular characterization of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II of *Acinetobacter calcoaceticus*. *Biochem. J.* **1998**, 330, 1375-1381.

(20) Shaw, J. P.; Rekik, M.; Schwager, F.; Harayama, S. Kinetic studies on benzyl alcohol dehydrogenase encoded by TOL plasmid pWW0. A member of the Zinc-containing long chain alcohol dehydrogenase family. *J. Biol. Chem*. **1993**, 268, 10842-10850.

(21) Collins, J.; Hegerman, G. Benzyl alcohol metabolism by *Pseudomonas putida*: a paradox resolved. *Arch. Microbiol*. **1984**, 138, 153-160.

(22) Chalmers, R. M.; Keen, J. N.; Fewson, C. A. Comparison of benzyl alcohol dehydrogenases and benzaldehyde dehydrogenases from the benzyl alcohol and mandelate pathways in *Acinetobacter calcoaceticus* and from the TOL-plasmid-encoded toluene pathway in *Pseudomonas putida*. *Biochem. J*. **1991**, 273, 99-107.

(23) Shaw, J. P.; Schwager, F.; Harayama, S. Substrate-specificity of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase encoded by TOL plasmid pWW0. Metabolic and mechanistic implications. *Biochem. J*., **1992**, 283, 789-794.

(24) Fan, F.; Lorenzen, J. A.; Plapp, B. V. An aspartate residue in yeast alcohol dehydrogenase I determines the specificity for coenzyme. *Biochemistry* **1991**, 30, 6397-6401.

(25) Keat, M. J.; Hopper, D. J. The aromatic alcohol dehydrogenases in *Pseudomonas putida* NCIB 9869 grown on 3,5-xylenol and *p*-cresol. *Biochem. J*. **1978**, 175, 659-667.

(26) Shaw, J. P; Harayama, S. Purification and characterization of TOL plasmid-encoded benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase of *Pseudomonas putida*. *Eur. J. Biochem*. **1990**, 191, 705-714.

(27) Chalmers, R. M.; Scott, A. J.; Fewson, C. A. Purification of the benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase encoded by the TOL plasmid pWW53 of *Pseudomonas putida* MT53 and their preliminary comparison with benzyl alcohol dehydrogenase and benzaldehyde dehydrogenases I and II from *Acinetobacter calcoaceticus*. *J. Gen. Microbiol*. **1990**, 136, 637-643.

(28) Sund, H.; Theorell, H. The enzymes, 2nd ed., pp. 25-38. Academic Press, New York, NY, **1963**.

(29) Curtis, A. J.; Shirk, M. C.; Fall, R. Allylic or benzylic stabilization is essential for catalysis by bacterial benzyl alcohol dehydrogenases. *Biochem. Biophys. Res. Commun*. **1999**, 259, 220-223.

(30) García-Moruno, E.; Ribaldone, M.; Di Stefano, R; Conterno, L.; Gandini, A. Study of five strains of *Saccharomyces cerevisiae* with regard to their metabolism towards geraniol. *J. Int. Sci. Vigne Vin*, **2002**, 36, 221-225.

(31) Overhage, J.; Steinbüchel, A.; Priefert, H. Biotransformation of eugenol to ferulic acid by a recombinat strain of *Ralstonia euthropha* H16. *Appl. Environ. Microbiol.* **2002**, 68, 4315-4321.

(32) Landete, J. M.; Rodríguez, H.; de las Rivas, B.; Muñoz, R. High-added-value antioxidants obtained from the degradation of wine phenolics by *Lactobacillus plantarum*. *J. Food Prot*. **2007**, 70, 2670-2675.

(33) Landete, J. M.; Curiel, J. A.; Rodríguez, H.; de las Rivas, B.; Muñoz, R. Study of the inhibitory activity of phenolic compounds found in olive products and their degradation by *Lactobacillus plantarum* strains. *Food Chem*. **2008**, 107, 320-326.

(34) Rodríguez, H.; Landete, J. M.; de las Rivas, B.; Muñoz, R. Metabolism of food phenolic acids by *Lactobacillus plantarum* CECT 748^{T}. Food Chem. **2008**, 107, 1393-1398.

(35) Rosillo, L.; Salinas, M. R.; Garijo, J.; Alonso, G. L. Study of volatiles in grapes by dynamic headspace analysis. Application to the differentiation of some *Vitis vinifera* varieties. *J. Chromatogr. A* **1999**, 847, 155-159.

(36) Bueno, J. E.; Peinado, R.; Moreno, J.; Medina, M.; Moyano, L.; Zea, L. Selection of volatile aroma compounds by statistical and enological criteria for analytical differentiation of musts and wines of two grape varieties. *J. Food Sci.* **2003**, 68, 158-163.

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FIGURE CAPTIONS

Figure 1. Comparison of benzyl alcohol dehydrogenase protein sequences from *Lactobacillus plantarum* (LPL) (accession Q88TC0), *Pseudomonas putida* (PPU) (accession P39849), *Acinetobacter* sp. ADP1 (AAD) (accession Q9XC27), and *Equus caballus* (ECA) (accession P00327). Clustal W program was used to compare predicted sequences. Residues conserved in all members of the Zn-containing alcohol dehydrogenases family (▼), residues involved in the interaction of NAD(P) (●), residue that makes a hydrogen bond to the substrate (♦), residues involved in zinc binding (○), or in the binding to the non-catalytic zinc (Δ) are indicated on the *L. plantarum* sequence. Residues that are identical (*), conserved (:) or semi-conserved (.) in all the sequences of the alignment are also indicated. Dashed represents gaps introduced to maximize similarities.

Figure 2. SDS-PAGE analysis of the expression and purification of the BADH protein from *L. plantarum* WCFS1. (A) Analysis of soluble cell extracts of IPTG-induced cultures.

Lane 1: *E. coli* JM109 (DE3) (pLysS) (pURI3). Lane 2: *E. coli* JM109 (DE3) (pLysS) (pURI3-BADH). (B) Analysis of fractions eluted after His-Trap-FF crude chelating affinity column (lines 1 to 4). BADH protein is indicated by an arrow. SDS-Polyacrylamide gels were stained with Coomassie blue. The positions of molecular mass markers (Bio-Rad) are indicated on the left.

Figure 3. Some biochemical properties of the *L. plantarum* WCFS1 purified BADH. (A) BADH activity as a function of temperature. (B) BADH activity as a function of pH. The experiments were done in triplicate. The mean value and the standard error are showed.

Table 1

Table 1. Effect of additives on *L. plantarum* WCFS1 BADH activity

Additions (10 mM)	Relative activity (%)
Control	100
KBr	111
KCl	100
NaCl	100
$MgCl_2$	122
$NiCl_2$	100
$CaCl_2$	127
$CdCl_2$	125
$AgNO_3$	42
Urea	100
Tween-80	100
DMSO	80
EDTA	78
β-mercaptoethanol	24
DTT	16
SDS	14

Table 2

Table 2. Relative activities of *L. plantarum* WCFS1 BADH with various potential substrates

Substrates (100 mM)	Relative activity (%)
Benzyl alcohol	100
Nerol	116
Geraniol	105
Phenethyl alcohol	73
Cinnamyl alcohol	67
Coniferyl alcohol	59
Tyrosol	0
Tryptophol	0
Pyrogallol	0
Catechol	0
Phloroglucinol	0
Ethanol	0
p-Coumaric acid	0
Caffeic acid	0

Figure 1

Figure 2

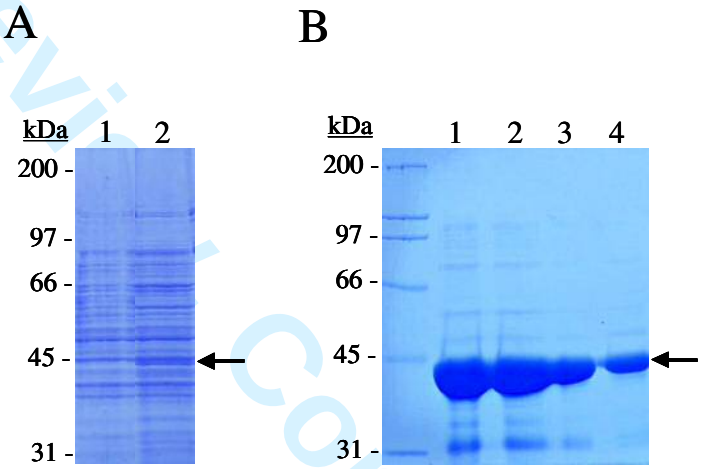

Figure 3