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Tannase activity by lactic acid bacteria isolated from grape
must and wine

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1 Abstract

2 We examined a range of oenological lactic acid bacteria species and reference
3 strains for their potential to degrade tannins. Bacterial tannase activity was checked by a
4 spectrophotometric and a visual reading method. None of the strains belonging to the
5 oenological species of the genus *Lactobacillus*, *Leuconostoc*, *Oenococcus* or
6 *Pediococcus* were tannase producers, with the exception of *Lactobacillus plantarum*.
7 All the *L. plantarum* strains analyzed were positive for tannase activity and their
8 identities were reconfirmed by a *L. plantarum* PCR-specific assay or by sequencing the
9 16S rDNA. Tannase activity could be considered an important criterion for the selection
10 of malolactic starter cultures since it might confer advantages in the winemaking
11 process by reducing astringency and haze in wine.

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16 *Keywords:* Tannase, Wine, Lactic acid bacteria, *Lactobacillus plantarum*, wine haze

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1. Introduction

2

3 Tannin acyl hydrolase (E.C. 3. 1. 1. 20), commonly called tannase, catalyzes the
4 hydrolysis of ester bonds in hydrolyzable tannins such as tannic acid, thereby releasing
5 glucose and gallic acid (Lekha and Lonsane, 1997). Tannins are defined as naturally
6 occurring water-soluble polyphenols of varying molecular weight depending on the
7 bonds possessed with proteins and polysaccharides (cellulose and pectin). Tannins are
8 widespread in the plant kingdom, are found in leaves, fruits, bark and wood.

9 Tannase finds widespread application in food and beverage processing. At the
10 moment most of the commercial applications of tannase are in the manufacturing of
11 instant tea –where it is used to eliminate water insoluble precipitates–, wine, beer and
12 coffee-flavored soft drinks. Other important application of tannase in the food industry,
13 is its use as substrate for the chemical synthesis of pyrogallol or ester galates, which are
14 used as preservatives. Gallic acid is also used in the enzymatic synthesis of propyl
15 gallate, which is mainly used as antioxidant in fats and oils, as well as in beverages
16 (Lekha and Lonsane, 1997).

17 In the case of wines, tannase hydrolyzed chlorogenic acid to caffeic acid and
18 quinic acid, which favorably influences taste (Lekha and Lonsane, 1997). Tannase has
19 been used along with laccase for the treatment of grape juice and grape musts to remove
20 phenolic substances for chemical stabilization of the beverage. Moreover, fifty percent
21 of the colour of the wine is due to the presence of tannins; however, if these compounds
22 are oxidized to quinones by contact with the air, they could form an undesirable
23 turbidity, which causes severe quality problems. The use of tannase has been proposed
24 as the best solution to this problem (Aguilar and Gutierrez-Sánchez, 2001).

1 Tannase can be obtained from plant, animal and microbial sources. The most
2 important source to obtain the enzyme is by microbial way, because the produced
3 enzymes are more stable than similar ones obtained from other sources (Bhat et al.,
4 1998). It has long been known that several fungal species such as *Aspergillus* spp.
5 (Banerjee and Mondal, 2001) and *Penicillium* spp. (Rajakumar and Nandy, 1983) are
6 capable of producing large amounts of tannase. Over the past decade, many bacterial
7 species have also been reported to produce tannase. These include *Streptococcus*
8 *gallolyticus* (Osawa et al., 1995a), *Lonepinella koalarum* (Osawa et al., 1995b), *Bacillus*
9 *licheniformis* (Mondal and Pati, 2000), and several lactobacilli species (Osawa et al.,
10 2000). However, there is no report describing the presence of tannin-degrading bacteria
11 in the wine-related microbiota in spite of the undoubted interest of this enzymatic
12 activity in the wine-making process.

13 The aim of this study was to examine the occurrence of tannase activity in
14 several strains of LAB isolated from Spanish grape musts and wines.

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17 2. Materials and methods

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19 2.1. *Strains and growth conditions*

20 Pure cultures of wine LAB control strains were provided by the Spanish Type
21 Culture Collection (CECT). A total of 78 LAB were obtained from the bacterial culture
22 collection of the Instituto de Fermentaciones Industriales (IFI), CSIC, Spain. These
23 strains were originally isolated from must grape or wine of different wine-producing
24 areas of Spain and classified by using biochemical tests (Moreno-Arribas et al. 2003).

1 Strains of *O. oeni* were grown on medium for *Leuconostoc oenos* (MLO medium)
2 (Caspritz and Radler, 1983) supplemented with 10% tomato juice. The other LAB
3 tested were grown in MRS broth (Difco, France). All bacteria were incubated at 30 °C
4 in a 5% CO₂ atmosphere.

5 6 7 2.2. Bacterial DNA extraction

8
9 Chromosomal DNA from selected LAB strains was isolated using the following
10 protocol. Briefly, the LAB strains were grown at 30 °C for 48 h in 10 ml of MRS broth.
11 **The cells were pelleted by centrifugation and resuspended in 600 µl of TE (10 mM Tris–**
12 **ClH pH 8.0, 1 mM EDTA) solution containing 10 mg/ml of lysozyme (Sigma,**
13 **Germany). The cells were lysed by adding 70 µl of 10% SDS (w/v) and 10 µl of**
14 **proteinase K (20 mg/ml) (Sigma, Germany). Crude DNA preparation was purified by**
15 **performing two phenol:chloroform:isoamyl alcohol (25:24:1) and one**
16 **chloroform:isoamyl alcohol (24:1) extractions. Chromosomal DNA was precipitated by**
17 **adding two volumes of cold ethanol. The precipitated DNA was washed with 70%**
18 **ethanol and left to air dry. The DNA pellet was dissolved in an appropriate volume of**
19 **TE buffer.**

20 21 22 2.3. *Lactobacillus plantarum* identification based on PCR

23
24 The PCR-specific reaction of *Lactobacillus plantarum* were performed using the
25 specific primers LbPI1 (5′-AATTGAGGCAGCTGGCCA-3′) and LbPI2 (5′-
26 GATTACGGGAGTCCAAGC-3′) described by Quere et al. (1997). These primers

1 amplified a 250 pb fragment in all the *L. plantarum* strains tested. This DNA fragment
2 revealed no homology to any known sequences contained in standard databases. PCR
3 **reaction was performed in 0.2 ml microcentrifuge tubes in a total volume of 25 μ l**
4 **containing 1 μ l of template DNA (aprox. 100 ng), 20 mM Tris-HCl, pH 8.0, 50 mM**
5 **KCl, 2.5 mM MgCl₂, 200 μ M of each dNTP, primer LbP11 (1 μ M), primer LbP12**
6 **(1 μ M) and 1 U of Ampli *Taq* DNA polymerase.** The reaction was performed in a
7 GeneAmp PCR System 2400 (Perkin Elmer) using the following cycling parameters:
8 initial 5 min denaturation at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 50
9 °C and 30 sec at 72 °C. Amplified products were analyzed by 2% agarose gel
10 electrophoresis in TAE buffer (Sambrook et al., 1989). The gel was stained with
11 ethidium bromide and the bands were visualized under UV illumination.

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14 2.4. PCR amplification of 16S rDNA

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16 16S rDNAs were PCR amplified using the eubacterial universal pair of primers 63f
17 (5'-CAGGCCTAACACATGCAAGTC-3') and 1387r (5'-
18 GGGCGGWGTGTACAAGGC-3') (W = A or T) previously described (Marchesi et al.,
19 1998). The 63f and 1387r primer combination generates an amplified product of 1.3 kb.
20 PCR reaction was performed as described above. PCR cycling parameters were: initial 5
21 min denaturation at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 50 °C and
22 1:30 min at 72 °C. Amplified products were resolved on a 0.7% agarose gel. The
23 amplifications products were purified on QIAquick spin Columns (Quiagen) for direct
24 sequencing.

25

1 2.5. DNA sequencing

2
3 DNA sequencing was carried out by using an Abi Prism 377TM DNA sequencer
4 (Applied Biosystems, Inc). Sequence similarity searches were carried out using Basic
5 local alignment search (BLAST) (Altschul et al., 1997) on the EMBL/GenBank
6 databases.

9 2.6. Tannase activity

10
11 Tannase activity of the isolates was tested following the spectrophotometric and
12 visual reading methods described by Osawa and Walsh (1993). Briefly, fresh cultures
13 on MRS or MLO agar plates were harvested with sterile cotton swabs and suspended in
14 1 ml of substrate medium (pH 5.0) containing 33mM NaH₂PO₄ and 20mM
15 methylgallate (Fluka Chemie, Germany) to prepare a dense suspension (at least
16 equivalent to a no. 3 McFarland turbidity standard). The substrate medium was then
17 incubated aerobically at 37 °C for 24 h. After incubation, the sample was alkalinized
18 with an equal amount of saturated NaHCO₃ solution (pH 8.6) and exposed to the
19 atmosphere at room temperature (23 °C) for 1 h. Green to brown coloration of the
20 medium was judged as a positive indicator of tannase activity in the visual reading
21 method. In the spectrophotometric method, 1ml of the suspension was removed,
22 centrifuged and the supernatant was read at A₄₄₀ in a spectrometer (S-22 UV/vis
23 spectrophotometer, Boeco, Germany).

3. Results and discussion

A number of microorganisms –including bacteria, fungi, and yeasts–have been reported to produce tannase. Extensive screening studies have been conducted to select potent cultures for tannase production. Bacterial species frequently isolated from wine were not included in previous studies. In this work, a wide range of wine lactic acid bacterial species was examined for tannase activity. In our study, several strains, including type strains, belonging to *L. mesenteroides* (11 strains), *Oenococcus oeni* (41 strains), pediococci (4 strains), *Lactobacillus buchneri* (9 strains), *L. fermentum* (1 strain), *L. fructivorans* (1 strain), *L. hilgardii* (8 strains) species were analysed for tannase activity. The tannase-positive *Lactobacillus plantarum* ATCC 14917 strain and tannase-negative *L. brevis* ATCC 8287 (CECT 4669) strain (Osawa et al., 2000) were used as reference strains in our screening.

Table 1 shows the number of positive strains of the total number of strains investigated. The tannase-negative strains by the visual method showed absorbance readings at 440 nm below 0.2 and the reaction media presented an light yellow colour (data not shown). However, absorbance values up to 0.3 were considered by Osawa and Walsh (1993) as negative for tannase production in two *Streptococcus bovis* strains analyzed. As could be observed from Table 1 tannase positive strains, including the positive control strain, showed a brownish colour of the media and presented absorbance values ranging from 0.716 to 0.980. In the study of Osawa and Walsh (1993) tannase positive enterobacterial strains gave absorbance values higher than 2.0; however, they considered also as positive absorbance values above 0.5.

In our study, identical results were obtained by using the spectrophotometric and the visual reading method in order to identify tannase-producing bacterial strains.

1 Therefore, as described previously (Osawa and Walsh, 1993), it might be concluded that
2 the visual reading method is a simple method for detection of bacterial tannase, it does
3 not require sophisticated analytic equipment and it can be incorporated into a
4 conventional test system for bacterial identification.

5 As deduced from Table 1, it is noteworthy that tannase-producing strains only
6 belongs to the *Lactobacillus plantarum* species. Previously, Osawa et al. (2000) isolated
7 lactobacilli with tannase activity from human feces and fermented foods. All the
8 tannase-producing isolates belong to the *L. plantarum*, *L. paraplantarum* and *L.*
9 *pentosus*. They examined for tannase activity a range of 14 different *Lactobacillus*
10 species obtained from culture collections. All the strains belonging to these 14 different
11 *Lactobacillus* species were negative for tannase activity.

12 Bacterial species frequently isolated from wine were not included in previous
13 studies. This is the first report on the tannase activity from strains belonging to
14 *Leuconostoc*, *Oenococcus* or *Pediococcus* genus. In the same way, there are not
15 tannase-activity studies of strains belonging to *L. buchneri*, *L. fermentum*, *L.*
16 *fructivorans* and *L. hilgardii* species, which are frequently isolated from wines.

17 In this study, all the strains presumptively classified as *L. plantarum* were positive
18 for tannase-activity. We used a previously described PCR-based method (Quere et al.,
19 1997) for the specific identification of *L. plantarum*. The method is designed to amplify
20 a 250 pb DNA fragment *L. plantarum* specific. We performed this PCR assay on total
21 DNA extracted from the presumptively identified *L. plantarum* strains (*L. plantarum*
22 ATCC 14917, and BIFI-31, 34, 35, 38, 39, 40, 41, 71, 72 and 73). Figure 1 showed the
23 results of the PCR amplification. As expected, all the strains analysed gave a 250 bp
24 DNA band specific of this species. The PCR results confirmed the classification of
25 these strains as members of the *L. plantarum* species.

1 Surprisingly, besides the *L. plantarum* isolates another LAB strain gave a
2 positive reaction for tannase activity. This strain, BIFI-28, was presumptively identified
3 as *Oenococcus oeni*. To confirm the taxonomical identity of this strain, a 1.3 kb DNA
4 fragment coding for the 16S rRNA was amplified. The bacterial isolate identified as
5 being positive for tannase activity was then identified using sequence data from the first
6 500 bp of the 16S rRNA gene. The sequence obtained was identified by database
7 comparison (BLAST search) using the GenBank nucleotide database (Altschul et al.,
8 1997). This isolate contained a sequence identical with that of *L. plantarum* strains
9 included in the databases. Therefore, the BIFI-28 isolate needs to be reclassified as *L.*
10 *plantarum* BIFI-28 (Table 1).

11 From these results it may be concluded that all the *L. plantarum* isolates
12 analyzed were the only tannase-producers strains. This result concurs with those of
13 Osawa et al. (2000), which showed that tannase activity is common in *L. plantarum*
14 strains. They postulated that this enzymatic property have an ecological advantage for
15 this specie, as it is often associated with fermentations of plant materials.

16 During winemaking process, lactic acid bacteria are responsible of the
17 malolactic fermentation (MLF). Since the beginning of the 1980s, commercial starter
18 cultures for the induction of MLF have been available, consisting of strains of *O. oeni*,
19 *L. plantarum* and *L. hilgardii* as single-or multiple-strain preparations. Tannase activity
20 could be considered an criterion for the selection of malolactic starter cultures
21 (Buckenhüskes, 1993) since it might represent several advantages in winemaking. First
22 of all, tannins are well known to interact with proteins. Clear beverages are generally
23 intended to remain clear until they are purchased and consumed. The most frequent
24 cause of haze in wine results from protein-polyphenol interaction (Siebert, 1999).
25 During vinification, a part of the soluble grape proteins is precipitated via interaction

1 with tannins. These precipitates are generated in a natural way when the beverage is
2 cooled at temperatures lower than 4 °C, and if this are removed chemically (employing
3 bentonite), a great amount of aromatic compounds can be eliminated. Tannase
4 hydrolyses the esters bonds from polyphenols, avoiding their polymerization, giving a
5 wine with a high content of aromatic compounds and appropriate colour, increasing its
6 quality (Aguilar and Gutierrez-Sánchez, 2001).

7 Besides, hydrolysable tannins extracted from the oak barrel during wine ageing,
8 are the main responsible of the wine astringency (Robichaud and Noble, 1990). Thus
9 interactions of tannins with proteins also occur in the oral cavity where salivary proteins
10 precipitate, giving rise to the mouth sensation of astringency. During storage and ageing
11 of wine, astringency decreases as these large molecules precipitated and the wine's
12 capacity to bind proteins fell. In a similar manner, the astringency could decrease
13 without any precipitate by the use of tannase that hydrolyses tannins present in the wine.

14 In summary, this is the first study reporting the occurrence of lactobacilli capable of
15 degrading hydrolyzable tannin in wine. *L. plantarum* was the only species showing
16 tannase activity. None of the other lactobacilli species or species from the genus
17 *Leuconostoc*, *Oenococcus* or *Pediococcus* were tannase-producers. Due to the potential
18 beneficial effects of tannase during winemaking, this activity could be considered as an
19 important criterion for the selection of malolactic bacterial starters.

20

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22

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14 Figure captions

15

16 Figure 1. PCR amplification of a specific–DNA fragment from *L. plantarum*.
17 Chromosomal DNA from the strains previously classified as *L. plantarum* were used for
18 PCR amplification with oligonucleotides LbPI1 and LbPI2. (1) *L. plantarum* ATCC
19 14917; (2) BIFI–31; (3) BIFI–34; (4) BIFI–35; (5) BIFI–38; (6) BIFI–39; (7) BIFI–40; (8)
20 BIFI–41; (9) BIFI–71; (10) BIFI–72; and (11) BIFI–73. Products were subject to agarose
21 gel electrophoresis and stained with ethidium bromide. Left lane, 50–bp Ladder.
22 Numbers indicate some of the molecular sizes (in bp).

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Table 1
Tannase-producing LAB strains isolated from grape must and wine

Name as collected	Strain	Source	Tannase		Taxon confirmed by PCR ^c or sequencing ^d	
			A ₄₄₀ ^a	Color ^b		
<i>Lactobacillus plantarum</i>	ATCC ^e 14917 ¹	Pickled cabbage	0.846	+	<i>L. plantarum</i> ^c	
	BIFI ^f -31	Wine	0.716	+	<i>L. plantarum</i> ^c	
	BIFI-34	Wine	0.825	+	<i>L. plantarum</i> ^c	
	BIFI-35	Wine	0.957	+	<i>L. plantarum</i> ^c	
	BIFI-38	Wine	0.768	+	<i>L. plantarum</i> ^c	
	BIFI-39	Wine	0.861	+	<i>L. plantarum</i> ^c	
	BIFI-40	Wine	0.736	+	<i>L. plantarum</i> ^c	
	BIFI-41	Wine	0.980	+	<i>L. plantarum</i> ^c	
	BIFI-71	Wine	0.811	+	<i>L. plantarum</i> ^c	
	BIFI-72	Wine	0.829	+	<i>L. plantarum</i> ^c	
	BIFI-73	Wine	0.845	+	<i>L. plantarum</i> ^c	
	<i>Oenococcus oeni</i>	BIFI-28	Wine	0.863	+	<i>L. plantarum</i> ^d

^a A₄₄₀, absorbance reading at 440 nm

^b +, green to brown tinging of the medium

^c PCR assay as described by Quere et al.(1997)

^d DNA sequencing of the 16S rDNA amplified using primers 63f and 1387r (see section 2.4)

^e ATCC, American Type Culture Collection

^f BIFI, Bacterial culture collection from the Instituto de Fermentaciones Industriales