

Isolation and Enological Characterization of Malolactic Bacteria from the Vineyards of Northwestern Spain

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Thirty-five strains of malolactic bacteria were isolated from grapes and alcoholic and malolactic fermentations in two vineyards from northwestern Spain. These belonged to six species of the genera *Lactobacillus* and *Leuconostoc*. The results of their partial enological characterization showed that 47.5% utilized more than 80% of the initial malic acid.

As a result of the climatic conditions affecting certain regions of northwestern Spain, low-maturity grapes are produced. This results in high malic acid concentrations both in must and wine. Bacterial malolactic fermentation is a suitable biological process for correcting wine acidity. It consists of malic acid decarboxylation, yielding lactic acid plus CO₂. This transformation is conducted by malolactic bacteria belonging to the genera *Lactobacillus*, *Leuconostoc*, and *Pediococcus* (2, 9, 18). At the same time, and on account of this secondary fermentation, new sensorial characteristics emerge (5). Although under the best conditions malolactic fermentation may take place spontaneously, in the studied regions, it rarely occurs. Additionally, some strains are inadequate for carrying out such a fermentation because they either exhibit low fermentative efficiency or produce undesirable compounds. In these cases, improvement of wine quality must rely upon the use of autochthonous and selected lactic acid bacteria to carry out the fermentations in a proper way.

Studies on the isolation and identification of lactic acid bacteria from wine have been carried out and reviewed by several authors in various regions of the world (14, 15, 18). Studies concerning the evolution of these microbial populations during the wine fermentation process have also been reported (3, 11, 14).

The present communication reports on the results of the isolation, identification, distribution, and partial enological characterization and potential of malolactic bacteria from grapes and from spontaneous alcoholic and malolactic fermentations studied at El Rosal and El Condado, two regions located in northwestern Spain.

Samples were collected aseptically in one cellar at El Rosal (C) and two cellars at El Condado (A and B) during 1988. Samples from grapes, sulfited must (stage I), active alcoholic fermentations (stage II), the end of the alcoholic fermentations (stage III), and malolactic fermentations (M.F.) were taken. Total bacterial colonies were determined in either diluted or concentrated samples, depending on the cellular density, in two culture media simultaneously: MRS (Oxoid Ltd.) (6), pH 5.5, supplemented with 100 mg of cycloheximide per liter (Sigma Chemical Co.) (3) and nutrient broth (BBL Microbiology Systems). Agar (Difco Laboratories) was added to a final concentration of 20 g/liter. The

cultures were then grown at 30°C for 5 days in an anaerobic jar supplemented with CO₂.

Identification of the isolated cultures was performed according to the criteria established in *Bergey's Manual of Systematic Bacteriology* (17). The following criteria and tests were considered for taxonomical analysis: cell morphology, Gram stain, catalase and oxidase production, fermentation of 23 different carbon sources (1), oxidation or fermentation of glucose, production of gas from glucose, indole production, spore formation, growth at different temperatures, growth in the presence of 10% ethanol, and degree of halotolerance. To confirm the obtained results, two reference strains belonging to the Spanish Type Culture Collection *Lactobacillus plantarum* (C.E.C.T. no. 220) and *Leuconostoc oenos* (C.E.C.T. no. 218) were included.

Characterization of the enological strains was carried out in microvinification assays. Bottles with 110 ml of wine, sterilized by filtration through 0.22- μ m filters (Millipore Corp.) and obtained by alcoholic fermentation of musts belonging to the same region as the strain to be tested, with a malic acid content of ca. 4.5 to 5.5 g/liter and pH of 3.5, were inoculated in each case with a final bacterial density of 10⁶ cells per ml (10, 15). These were incubated under anaerobic conditions for 7 days at 21 to 23°C (15). Wine samples were taken at the beginning and at the end of the fermentation, sterilized as before and analyzed for malic, lactic, and acetic acid contents by enzymatic tests (Boehringer Mannheim Biochemicals).

Isolation of strains. In the two regions studied, 148 bacterial strains were isolated; 35 of these were lactic acid bacteria (23.6%). Of the latter strains, 3 belonged to cellar A, 24 belonged to cellar B, and 8 belonged to cellar C, with 27 strains corresponding to El Condado and 8 to El Rosal. Table 1 shows the comparative percentages of isolation in the three cellars sampled during the successive steps of the winemaking process.

Appreciable difficulties arose in the isolation of malolactic bacteria from grapes, where bacterial density is low. In fact, they were isolated only in cellar C (25%). This result may be explained by the fact that the regions studied have a high rainfall and the grapes are thus subject to a washing effect (1).

During active fermentation (stage II), no malolactic bacteria were isolated. This may be explained in terms of competition with yeasts, which at that time were in the log phase of growth and were actively excreting several prod-

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TABLE 1. Isolation of malolactic bacteria from grapes and from alcoholic and malolactic fermentations in the two vineyards studied (El Condado, cellars A and B, and El Rosal, cellar C)

Cellar	Species	No. (%) isolated from the following ^a :				
		Grapes	Stage I	Stage II	Stage III	M.F.
A	<i>Lactobacillus plantarum</i>				2 (100)	
	<i>Leuconostoc oenos</i>					1 (100)
	Total				2 (66.7)	1 (33.3)
B	<i>Lactobacillus plantarum</i>		5 (24)		7 (33)	9 (43)
	<i>Lactobacillus casei</i>				1 (100)	
	Total		5 (21)		7 (29)	12 (50)
C	<i>Lactobacillus plantarum</i>				1 (33)	2 (67)
	<i>Lactobacillus curvatus</i>	1 (50)				1 (50)
	<i>Lactobacillus buchneri</i>	1 (100)				
	<i>Lactobacillus brevis</i>		1 (100)			
	<i>Leuconostoc oenos</i>					1 (100)
	Total	2 (25)	1 (12.5)		1 (12.5)	4 (50)

^a Stage I, sulfited must; stage II, active alcoholic fermentations; stage III, the end of the alcoholic fermentations; M.F., malolactic fermentation.

ucts, such as ethanol (8, 15), SO₂ (7), and octanic and decanoic acids (12), which inhibit bacterial growth (2, 7).

As expected, the stage corresponding to M.F. showed the highest percentages of isolation (33.3 to 50%). During this period, the growth of malolactic bacteria was helped by the increase in yeast lysis products (2). It should be noted that in cellar A, the highest percentages of malolactic bacteria were isolated at the end of the alcoholic fermentation (stage III).

Taxonomical analysis. The 35 isolates were grouped into six species and belonged to the genera *Lactobacillus* and *Leuconostoc*; no strains belonging to the genus *Pediococcus* were isolated. Species of this latter genus are frequently isolated from other regions of the world (3, 14, 18).

In cellar A (El Condado), only strains of the species *L. plantarum* and *L. oenos* were isolated. The percents and distribution values are shown in Table 1. In cellar B, in addition to the two above-mentioned species, *Lactobacillus casei* was also recovered. Finally, cellar C (El Rosal) showed the highest variation in the number of species isolated. This was the only case in which isolations from grapes were positive (*Lactobacillus curvatus* and *Lactobacillus buchneri*).

Enological characterization. The results obtained are summarized in Table 2. Only one strain was unable to ferment malic acid, whereas the rest showed a malolactic activity ranging between 18.2 and 99.5%. A total of 16 of the 35 strains assayed (45.7%) had fermentation values higher than 80%, and these were mainly isolated during malolactic fermentation. The only two strains with malolactic activities lower than 20% were isolated from grapes.

The efficiency of lactic acid formation from malic acid was highly variable, with values between 34.2 and 100% (57% of strains). Great variability was also observed among strains of the same species in terms of their malolactic activity (15): in this sense, it may be inferred that this characteristic depends more on the strain than on the species. It should be noted that the two reference strains used showed poor malolactic activity, probably due to their difficulties in adapting to the media.

Malolactic fermentation may produce an undesirable increase in the acetic acid content (0.1 to 0.2 g/liter) (15). Because of this, the strains used for conducting the fermentation must be negatively selected for this character. With the strains reported here, values of between 0.01 and 0.8 were obtained. This was probably due to higher levels of

TABLE 2. Enological characterization of the malolactic bacteria isolated

Strain/stage	Species	Malic acid consumed (%)	Lactic acid yield (%)	Increase in acetic acid (g/liter)
1/III	<i>L. plantarum</i>	68.4	100	0.15
2/III	<i>L. plantarum</i>	68.4	100	0.04
3/M.F.	<i>L. oenos</i>	95.9	100	0.20
4/I	<i>L. plantarum</i>	31.6		
5/III	<i>L. plantarum</i>			
6/III	<i>L. plantarum</i>	22.4	34.9	
7/III	<i>L. plantarum</i>	28.6		
8/III	<i>L. plantarum</i>	60.4	64.7	
9/M.F.	<i>L. plantarum</i>	91.2	100	0.06
10/M.F.	<i>L. casei</i>	95.5	100	0.29
11/M.F.	<i>L. plantarum</i>	20.3	100	
12/M.F.	<i>L. plantarum</i>	68.4	100	0.15
13/M.F.	<i>L. plantarum</i>	85.9	91.7	0.00
14/M.F.	<i>L. casei</i>	82.0	100	
15/M.F.	<i>L. plantarum</i>	37.5		
16/M.F.	<i>L. plantarum</i>	83.4	100	0.04
17/M.F.	<i>L. plantarum</i>	30.7		
18/M.F.	<i>L. plantarum</i>	95.4	100	0.06
19/M.F.	<i>L. plantarum</i>	82.7	98.9	0.00
20/I	<i>L. plantarum</i>	66.6	100	0.11
21/I	<i>L. plantarum</i>	62.7	100	
22/I	<i>L. plantarum</i>	73.2	100	0.05
23/III	<i>L. plantarum</i>	52.7	100	0.13
24/III	<i>L. plantarum</i>	83.4	100	0.03
25/I	<i>L. plantarum</i>	99.5	100	0.04
26/M.F.	<i>L. plantarum</i>	95.5	100	0.02
27/M.F.	<i>L. oenos</i>	87.2	94.4	0.13
28/II	<i>L. plantarum</i>	99.4	100	0.03
29/M.F.	<i>L. curvatus</i>	84.5	100	0.09
30/M.F.	<i>L. plantarum</i>	92.3	94.6	0.01
31/M.F.	<i>L. plantarum</i>	21.5	87.1	0.15
32/Grapes	<i>L. buchneri</i>	18.2	34.2	0.13
33/Grapes	<i>L. curvatus</i>	18.2	34.2	0.13
34/I	<i>L. brevis</i>	57.2	100	0.01
35/M.F.	<i>L. oenos</i>	81.2	81.1	0.80
CECT no. 220	<i>L. plantarum</i>	8.80	12.4	
CECT no. 218	<i>L. oenos</i>	21.7	21.6	

citric acid (4, 13, 16), also present in this type of wine (data not shown). Among the strains exhibiting the highest activities as well as fermentative efficiencies, only three (strains 3, 10, and 35, respectively), showed considerable increases in their acetic acid levels. These were two heterofermentative strains of *L. oenos* and one strain of *L. casei*.

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