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Research Note

Biogenic Amine Production by *Oenococcus oeni* Isolates from Malolactic Fermentation of Tempranillo Wine

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ABSTRACT

In this article, we examine the production of biogenic amines, histamine, putrescine, tyramine, and cadaverine by 90 strains of *Oenococcus oeni* isolated from different cellars of Castilla-La Mancha (Spain) during wine malolactic fermentation. Amino biogenic capacity of strains was qualitatively analyzed on agar. After that, production of amines on a synthetic medium and on wine, and presence in strains of histidine, ornithine, and tyrosine decarboxylase genes were determined. Only two strains were able to produce histamine or putrescine, both on synthetic medium and wine. The presence of the corresponding genes in these strains was also confirmed. These results suggest that *O. oeni* does not significantly contribute to the overall biogenic amine content of wines. The main contribution of this work is the isolation of a putrescine-producing *O. oeni* strain that harbors the ornithine gene, since this gene appears to be rarely present in the genome of *O. oeni.*

Biogenic amines are compounds formed by decarboxylation of amino acids, with participation of substrate-specific enzymes from diverse microorganisms *(27),* which can cause toxicological problems even if relatively low amounts are ingested.

In wine, it has been reported that the concentration of biogenic amines increases during malolactic fermentation (MLF) *(23),* and therefore, studies have been conducted on production of biogenic amines, especially histamine, by lactic acid bacteria (LAB) participating in MLF *(8, 11).* In these studies, the authors report that the aminobiogenic capacity of LAB strains used as starter cultures for MLF is an interesting property that should be tested prior to selection of the strains.

Several qualitative and quantitative methods to determine the production of biogenic amines have been described *(2, 9, 17).* The most common screening procedures involve the use of a differential medium containing a pH indicator such as bromocresol purple, although some authors have reported false-positive reactions when these media are used, due to the formation of alkaline compounds other than biogenic amines *(22).* Therefore, the use of complementary tests has been recommended, such as the detection of targeted genes by specific PCR. Conditions used in the detection of amino acid decarboxylase genes are reported elsewhere *(4, 5, 13, 15, 18).*

The aim of this study was to assess the occurrence of amino acid decarboxylase activities of 90 strains of *Oenococcus oeni* isolated from samples of Tempranillo wine taken at different stages of MLF from nine cellars situated in the Castilla-La Mancha region (Spain), using qualitative and quantitative methods. In addition, in order to study the biogenic amine production in wine, strains were inoculated in wine once alcoholic fermentation had finished. The amine concentrations produced by *O. oeni* in wine were determined by liquid chromatography.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A total of 90 *O. oeni* isolates were studied. They were representative of the main genotypes identified after random amplification of polymorphic DNA–PCR analysis, using primer M13 and conditions described by Ruiz et al. *(26),* of 446 isolates obtained from Tempranillo wine samples. Strains were grown on medium for *Leuconostoc oenos* (MLO; Scharlab, Barcelona, Spain) agar plates that were incubated at 28°C under anaerobic conditions (AnaeroGen, Atmosphere Generation System, Oxoid, Ltd., Basingstoke, Hampshire, UK). They were propagated twice in MLO broth before using. A culture of *Lactobacillus* 30a known to produce histamine and putrescine was used as a positive control for the specific PCR reactions.

Determination of biogenic amine-forming capacity on plates. Amino acid decarboxylase activity of all 90 strains of *O. oeni* was assessed by using the decarboxylase medium described by Maijala *(17).* Two percent (wt/vol) of each precursor amino acid (L-histidine monohydrochloride, L-ornithine monohydrochloride, tyrosine disodium salt, and L-lysine monohydrochloride, all purchased from Sigma [St. Louis, MO]), and 0.005% (wt/vol) pyridoxal-5-phosphate (as a cofactor for the decarboxylation reaction) were added to the medium. Plates were incubated at 28°C,

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under anaerobic conditions for 7 days. Positive reactions were recorded by a change of the medium color to purple or by a disappearance of tyrosine precipitates around the colonies *(2).*

Production of biogenic amines in synthetic medium. Positive and weakly positive strains from qualitative analysis were grown on MLO broth until an optical density at 600 nm of 0.9 was reached, and then they were inoculated at 1% (vol/vol) into Maijala medium *(17)* containing L-histidine monohydrochloride, L-ornithine monohydrochloride, tyrosine disodium salt, or L-lysine monohydrochloride. After incubation at 28°C for 7 days under anaerobic conditions, 2 ml of broth was centrifuged (13,000 × *g* for 5 min), and the cell-free supernatant was collected and stored at −20°C until analysis. Samples were analyzed by high-performance liquid chromatography (HPLC), according to the method described below.

Detection of the histidine decarboxylase, ornithine decarboxylase, and tyrosine decarboxylase genes. Genomic DNA from a single colony on MLO agar was extracted by following the procedure described by Rodas et al. *(25).* Presence of histidine decarboxylase (*hdc*), ornithine decarboxylase (*odc*), and tyrosine decarboxylase (*tdc*) genes was assayed by specific PCR reactions by following the procedure described by Marcobal et al. *(19).* Primer sets JV16HC and JV17HC *(13),* 3 to 16 *(19),* and P1-rev and P2-for *(15)* were used to detect *hdc, odc,* and *tdc* genes, respectively.

Production of biogenic amines in wine. A volume of 250 ml of Tempranillo wine was inoculated (1%, vol/vol) with each of the 90 strains. Flasks were aerobically incubated at 23°C, without shaking, until completion of MLF, as determined by the concentration of L-malic acid. After that, concentrations of amino acids (histidine, arginine, ornithine, tyrosine, and lysine) and biogenic amines (histamine, cadaverine, putrescine, and tyramine) were determined as described below. Each strain was assayed in duplicate. A sample of noninoculated wine was used as a blank.

The Tempranillo wine used had been fermented by using a commercial starter (UVAFERM VN Lallemand, Madrid, Spain) and after completion of the alcoholic fermentation, filtered through a 0.2-μm-pore-size module (Millipore, Billerica, MA) to sterilize. Chemical composition of wine was determined as described below.

Before inoculation, strains were grown in MLO broth with 10% (vol/vol) tomato juice and anaerobically incubated at 28°C until a population of around 10^6 viable cells per milliliter was reached.

Chemical analysis of wine. The most common physicochemical parameters in wine—alcoholic degree, pH, total acidity, volatile acidity, citric acid content, and SO_2 concentration—were determined by following the procedures described in the European Union *Commission Regulation Determining Community Methods for the Analysis of Wine (6).* Commercial enzymatic kits (Boehringer-Mannheim, Manheim, Germany) were used to determine L-malic and L-lactic acid contents.

Amino acid and biogenic amine determinations in wine by HPLC. Amino acid (histidine, arginine, ornithine, tyrosine, and lysine) and biogenic amine (histamine, cadaverine, putrescine, and tyramine) contents were determined by liquid chromatography by following the method proposed by Gómez-Alonso et al. *(7).* A Varian ProStar HPLC (Varian, Inc., Walnut Creek, CA) was used, equipped with a ProStar 240 pump, a ProStar 410 autosampler, and a ProStar 330 photodiode array detector. Separated compounds were identified based on the aminoenone-derivative retention times of the corresponding standard (Sigma-Aldrich Chemie, Steinheim, Germany) and quantified by using the internal standard method.

RESULTS AND DISCUSSION

Positive results on agar plates containing the precursor amino acids were recorded for 27 of the 90 strains studied. Nevertheless, only the strains B10L9, B10L10, D23L15, D12L8, J34L5, and E20L6 grew abundantly on the plates containing L-histidine, and strain J20L8 did so on the plates containing ornithine, probably because of the higher levels of energy obtained from decarboxylation of amino acids *(21).* None of the assayed strains produced changes on tyrosine or lysine plates.

When cell-free supernatants of cultures in Maijala broth of the 27 strains were analyzed by HPLC, production of histamine and putrescine by strains J34L5 and J20L8, respectively, was confirmed (data not shown), while no amine production was detected for the remaining strains.

Specific PCR reactions also confirmed these results and, in line with reports by some authors *(13, 19),* the presence of a 367-bp band, corresponding to the *hdc* gene *(13),* and a 1,446-bp band, corresponding to the *odc* gene *(19),* was observed at strains J34L5 and J20L8, respectively. According to the PCR results, none of the remaining strains were positive.

Moreno-Arribas et al. *(22)* also reported false-positive results from biogenic amine analysis on agar plates, and Lonvaud-Funel and Joyeux *(14)* described that LAB can lose the ability to produce biogenic amines after prolonged storage or cultivation in synthetic media. More recently, Lucas et al. *(16)* demonstrated that the *hdc* gene is located on an unstable plasmid, and that the ability to produce histamine could be lost, depending on culture conditions.

Different results have been reported for biogenic amines production by *O. oeni.* For histamine, discrepancies have been found, and while some authors reported no histamine production in the assayed *O. oeni* cultures *(3, 22),* others found that the presence of *O. oeni* histamine-producing strains is not rare *(5, 8, 11).*

The results of putrescine for strain J20L8 are interesting in that until now, the only LAB reported to possess the *odc* gene were *O. oeni* BIFI-83, *Lactobacillus* 30a, and *Lactobacillus hilgardii X1B (3, 11).* This fact is especially surprising, considering that putrescine is the most prevalent amine in wines *(10, 11).* It suggests either that other organisms would be responsible for putrescine production during MLF, or that it is produced through alternative pathways that do not require the presence of the *odc* gene *(1, 20).*

As has already been emphasized by some authors *(3, 11, 22),* a low diffusion of tyramine-producing ability was observed within *O. oeni* strains assayed in this study. This activity seems to be more frequent in *Lactobacillus* spp.

In concordance with our results for lysine, no references reporting the existence of cadaverine-producing LAB strains, including *O. oeni,* have been found. Lysine decarboxylase activity has been reported to be frequent in *Enterobacteriaceae* spp. *(24).*

TABLE 1. *Effect of LAB strain on wine biogenic amines content after MLF*

	Histamine (mg/liter)		Putrescine (mg/liter)		Tyramine (mg/liter)		Cadaverine (mg/liter)	
Strain	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Blank	0.23	0.10	4.94	0.52	0.70	0.07	0.36	0.04
A13L2	0.42	0.01	5.39	0.01	1.12	0.04	0.39	0.02
B21L6	0.27	0.01	4.67	0.18	0.73	0.04	0.33	0.00
B12L4	0.32	0.03	5.16	0.37	0.82	0.10	0.37	0.03
B13L4	0.29	0.01	5.08	0.17	0.88	0.04	0.36	0.01
B23L11	0.27	0.01	5.06	0.04	0.89	0.00	0.35	0.00
B23L9	0.17	0.03	5.26	0.01	0.84	0.05	0.40	0.00
C21L3	0.28	0.06	5.42	0.00	0.85	0.04	0.39	0.00
C22L9	0.38	0.02	5.38	0.03	0.86	0.05	0.39	0.01
C13L12	0.37	0.01	5.41	0.06	0.87	0.02	0.39	0.01
C12L5	0.43	0.01	5.45	0.04	0.94	0.08	0.40	0.01
D23L15	0.43	0.05	5.37	0.09	1.06	0.02	0.39	0.00
D23L6	0.40	0.00	5.30	0.02	0.80	0.04	0.38	0.00
D21L8	0.26	0.00	4.95	0.77	0.71	0.11	0.37	0.07
D13L13	0.39	0.01	5.36	0.00	0.91	0.09	0.39	0.02
E20L2	0.26	0.22	5.11	0.35	0.78	0.07	0.39	0.03
E11L7	0.17	0.06	4.04	0.83	0.62	0.16	0.31	0.07
F14L10	0.46	0.03	5.44	0.08	0.86	0.05	0.40	0.01
F10L4	0.12	0.06	3.99	1.64	0.59	0.26	0.30	0.13
G13L4	0.11	0.00	3.42	0.24	0.48	0.08	0.30	0.03
G13L3	0.29	0.04	5.39	0.02	0.95	0.09	0.41	0.01
G23L5	0.14	0.05	3.82	1.22	0.65	0.24	0.28	0.10
I24L7	0.39	0.01	5.44	0.05	0.81	0.03	0.39	0.01
I21L1	0.14	0.10	3.31	2.39	0.52	0.37	0.24	0.17
J24L7	0.34	0.10	5.36	0.11	0.80	0.11	0.38	0.02
J20L8	0.19	0.08	12.62	1.78	0.70	0.14	0.63	0.11
J34L5	9.24	0.91	4.50	0.26	0.73	0.09	0.33	0.02
J31L5	0.41	0.00	5.40	0.03	0.80	0.04	0.38	0.01

Chemical analysis of the Tempranillo wine used to assay production of amines in wine reported the usual values for these wines: 13.45% (vol/vol) alcohol content, 2.37 g/liter malic acid, 0.34 g/liter citric acid, 45 mg/liter total SO_2, 0.31 g/liter volatile acidity, 5.27 g/liter total acidity, and pH 3.59. Concentrations of amino acids in noninoculated wine, as determined by HPLC, were 14.62 mg/liter histidine, 43.49 mg/liter arginine, 17.03 mg/liter ornithine, 9.67 mg/liter tyrosine, and 25.05 mg/liter lysine.

Biogenic amine concentrations in wines inoculated with each of the 27 strains are shown at Table 1. Production of histamine and putrescine by strains J34L5 and J20L8, respectively, was again confirmed, and it is important to highlight that the amount of histamine produced was higher than the upper limits recommended in some countries. The quantity of amines formed by the remaining strains was negligible, with concentrations of cadaverine $\leq$0.40 mg/liter for all except strain J20L8, and concentrations of tyramine ranging between 0.5 and 1.1 mg/liter. Landete et al. *(12)* reported similar results, finding cadaverine concentrations in wine less than 0.5 mg/liter.

TABLE 2. *Effect of different LAB strains on wine biogenic amines and their precursor amino acids content*

Amino acid	Blank	J20L8	J34L5
Histidine	14.62	13.55	2.15
Arginine	43.49	38.68	42.59
Histamine	0.15	0.19	9.24
Ornithine	17.03	1.33	16.26
Putrescine	5.31	12.62	4.50

A correlation between biogenic amine and corresponding precursor amino acid concentrations was observed in wines inoculated with strains J34L5 and J20L8 (Table 2). In addition, for strain J20L8, a slight decrease in the arginine concentration occurred, which could have contributed to the formation of both ornithine, via the arginine deaminase pathway, and putrescine.

Like Landete et al. *(11, 12),* we found a good correlation between results for biogenic amine production in MLO broth and wine and presence of *hdc, odc,* and *tdc* genes.

Given the small number of strains of *O. oeni* that were able to produce biogenic amines, it is possible to state that *O. oeni* does not significantly contribute to the overall biogenic amine content of wines. In addition, the ability to produce the biogenic amines histamine, tyramine, putrescine, and cadaverine seems to be not a consistent characteristic of this species, but rather a strain-dependent property limited to a small number of strains.

The most relevant contribution of this work is the isolation of a putrescine-producing *O. oeni* strain, which har-

bors the *odc* gene since, as reported by numerous authors, the presence of the *odc* gene appears to be rarely present in the genome of *O. oeni.* Further research is being carried out to sequence this gene.

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