SHORT COMMUNICATION

Screening of biogenic amine production by lactic acid bacteria isolated from grape must and wine

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Abstract

The potential to produce the biogenic amines tyramine, histamine and putrescine, was investigated for lactic acid bacteria (LAB) of different origin, including commercial malolactic starter cultures, type strains and 78 strains isolated from Spanish grape must and wine. The presence of biogenic amines in a decarboxylase synthetic broth was determined by reversed–phase high performance liquid chromatography. Tyramine was the main amine formed by the LAB strains investigated. *Leuconostoc* strains were the most intensive tyramine formers. No potential to form biogenic amines was observed in *Oenococcus oeni* strains. Two strains of *Latobacillus buchneri* were associated with putrescine formation. None of the lactic acid bacteria produced histamine. According to these *in vitro* results, the commercial starter bacteria analyzed did not produce histamine, tyramine and putrescine.

Keywords: Biogenic amines, Amino acid–decarboxylase, Wine, Must, Lactic acid bacteria, HPLC

1. Introduction

Biogenic amines have been implicated in food poisoning incidents, usually from the consumption of fermented foods like cheese, meat, fish products and wine (Silla, 1996). Biogenic amines in food are mainly formed by decarboxylation of the corresponding amino acids by microorganisms. Musts and wines are very selective media, which can support growth of only few species of lactic acid bacteria (LAB). Four genera are represented: *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus*. During alcoholic fermentation, the LAB population is mainly composed of pediococci along with *Oenococcus oeni*. The homofermentative lactobacilli, the major type present on grapes, disappear quickly after the start of alcoholic fermentation in favor of *Leuconostoc mesenteroides* which at the end of the fermentation is replaced by *Oenococcus oeni* (Lonvaud–Funel, 1999).

In wine, several amino acids can be decarboxylated; as a result biogenic amines are usually found, with histamine, tyramine and putrescine being the most frequent. Formation of these amines in wines has been associated with a lack of hygiene during the winemaking process and it is generally believed that the formation of histamine in wines is due to spoilage bacteria, mainly *Pediococcus* spp. (Aerny, 1985; Delfini, 1989) and not to *O. oeni*. In 1990, Choudhury et al. showed that a strain of *O. oeni* (*O, oeni* DSM 20252), the main LAB responsible for malolactic fermentation, was able to produce tyramine in a laboratory medium. In 1994, *O.oeni* 9204 able to produce histamine, via histidine decarboxylase, was isolated from wine (Lonvaud–Funel and Joyeux, 1994). More recently, *Lactobacillus brevis* strains were associated with tyramine formation in wine (Moreno–Arribas and Lonvaud–Funel, 1999; Moreno–Arribas et al., 2000) and the tyrosine decarboxylase responsible was then purified and

characterized (Moreno–Arribas and Lonvaud–Funel, 2001). However, more research is required to correlate amine production in wine with species of LAB involved in winemaking process.

The aim of this study was to examine the occurrence of amino acid–decarboxylase activity of several strains of LAB isolated from Spanish grape must and wines as well as some commercial malolactic starters.

2. Materials and methods

2.1. Strains and growth conditions

The origin of each bacterial strain used in this study is shown in Table 1. Two pure cultures of LAB control strains were provided by the Spanish Type Culture Collection (CECT), including the tyramine–producing strain *Lactobacillus brevis* 5354 (ATCC 367). *Lactobacillus* 30a, a histamine and putrescine producing strain, was purchased from the American Type Culture Collection (ATCC). Four additional *O.oeni* strains were isolated from commercial malolactic starter preparations as described below, on MLO agar (Adsa, Spain) and selecting individual colonies. A total of 78 LAB were obtained from the bacterial culture collection of the Instituto de Fermentaciones Industriales (IFI), CSIC, Spain. These strains originally were isolated from must grape or wine of different wine–producing areas of Spain.

Strains of *Oenococcus oeni* were grown on Medium for *Leuconostoc oenos* (MLO medium) (Caspritz and Radler, 1983) supplemented with 10% tomato juice. The other LAB tested were grown in MRS broth (Difco, France). All bacteria were incubated at 30ºC in a 5% CO_2 atmosphere.

2.2. Qualitative detection of amine formation in decarboxylase assay medium

The strains were grown as indicated above. Production of biogenic amines was tested by inoculating each strain in the modified decarboxylase medium described by Maijala (Maijala, 1993). Pyridoxal–5–phosphate was included in the medium (at 0.005%) since its presence as a cofactor for the decarboxylation reaction has a strong enhancing effect on the amino acid decarboxylase activity (Recsei et al., 1985). The medium contained the corresponding precursor amino acid (L–histidine monohydrochloride, tyrosine di–sodium salt, L–ornithine monohydrochloride and L–arginine monohydrochloride) at a 0.5% final concentration, and purple bromocresol as pH indicator. The pH was adjusted to 5.3 and the medium was autoclaved. The precursor amino acids were purchased from Sigma (St. Louis, MO, USA).

A bacterial suspension (10^9 cfu/ml) was made from a plate culture in decarboxylase medium without amino acids incubated for 2–5 days at 30ºC. An aliquot of the suspension (0.2 ml) was inoculated into 2 ml of the same medium with and without amino acids as control. After 7 days incubation at 30ºC under anaerobic conditions by overlaying with paraffin, the medium was centrifuged and the supernatant was kept at –20ºC until analysis for biogenic amines.

2.3.Quantitative detection of biogenic amine producers

Analysis was carried out by reverse–phase high performance liquid chromatography (RP–HPLC) using a Waters liquid chromatograph controlled by the Millenium[32] program (Waters Corporation, Milford, Massachusetts). Samples were submitted to an

automatic precolumn derivatization with *o*-phthaldialdehyde (OPA), prior to injection, with reactant solution 100–150 fold higher in concentration than the BA. All separations were carried out on a Waters Nova-Pak C_{18} column (150 × 3.9 mm i.d., 60Å, 4 μm). Eluent and gradient conditions were similar to those described by Pereira-Monteiro and Bertrand (1994). Detection was by fluorescence using a Waters 420 fluorescence detector (340 nm excitation filter and 425 nm long-pass emission filter). Samples were injected in duplicate onto the column after being filtered through a 0.45 μm filter (Millipore, Bedford, MA, USA).

3. Results and discussion

Cultures of 85 strains representing 9 species of LAB were investigated for their potential to form histamine, tyramine and putrescine. Table 2 shows the number of positive strains of the total number of strains investigated.

Several qualitative and quantitative methods to determine production of biogenic amines by microorganisms have been described. Most of the screening procedures generally involve the use of a differential medium containing a pH indicator. A positive result is indicated by a change to purple in response of the indicator to a pH shift. The pH change is dependent on the production of the more alkaline amine from the amino acids initially included in the medium. Modifications to these media have been reported in order to adapt the method for different applications. In order to facilitate the growth of meat LAB, Maijala (1993) developed a modified decarboxylase media. Although the author reported the suitability of the media to determine production of biogenic amines by LAB, no *O.oeni* strain was tested. In our screening we used modified decarboxylase broth supplemented with pyridoxal-5-phosphate as a cofactor for the decarboxylase

reaction. This modification was successfully used by Bover–Cid and Holzapfel (1999) in a decarboxylase screening medium described previously. We tested a large number of fastidious LAB, including *O.oeni* strains, and all were able to growth in this medium. Biogenic amine–positive reactions were recorded when a purple color formed in the decarboxylase broth as result of LAB metabolism. We evaluated the suitability of the designed medium by confirmation of the quantitative amine–forming capacity using a RP–HPLC assay.

Results of biogenic amine production by control strains are also shown in Table 2. *Lactobacillus* 30a, has been described as producing high levels of histamine (Valler et al., 1982) and putrescine (Guirard and Snell, 1980), and *L. brevis* ATCC 367 (CECT 5354) of tyramine (Moreno–Arribas and Lonvaud–Funel, 1999). In our study, *Lactobacillus* 30a showed histidine decarboxylase activity and produced 1306 mgl^{-1} of histamine, and also had the highest ornithine decarboxylase activity detected (971 mgl^{-1} of putrescine). Of particular interest was the control strain *O.oeni* DSM 20252 (ATCC 23279, CECT 4100) that was described to produce significant quantities of putrescine and tyramine in fermented carrots, and later, when it was grown in a synthetic decarboxylase assay medium, only tyramine was produced (Choudhury et al., 1990). In our study, no potential was found in this strain to form any of the biogenic amines analyzed. This result concurs with those of Coton et al. (1998) showing that *O.oeni* ATCC 23279 did not produce amines when cultivated in a media containing 2 gl^{-1} of each precursor amino acid.

Straub et al. (1995) did not report any potential to form biogenic amines after analyzing 88 strains of *O. oeni*, the most important species in wine. In our study, among the oenococci tested, we did not find any producer of biogenic amines. In the tyrosine–decarboxylase assay, 3 out 39 oenococci gave a faint purple color to the media, but none

of them was confirmed by HPLC. This is not surprising since previous reports (Roig–Sagués et al., 1997) have described the occurrence of false–positive reactions, due to the formation of other alkaline compounds. In a previous study, Moreno–Arribas et al. (2000) analyzed wine containing high levels of biogenic amines in order to investigate the presence of tyramine–producing strains. They isolated two different *L. brevis* strains, none of the tyramine–producing strains were identified as *O. oeni*. As far as the literature suggests, the isolation of a tyramine–producing *O.oeni* strain from wine has not been reported.

In a survey of 118 wines randomly chosen in different wine–producing areas of South–West France, Coton et al. (1998) found that the presence of histamine–producing bacteria is not rare, as almost half of the tested wines possessed bacteria carrying the histidine decarboxylase gene and all of the strains belonged to *O. oeni*. In contrast with these results, formation of histamine was not observed in any species that may be involved in malolactic fermentation (Straub et al., 1995). In our screening, no histamine production was observed in cultures of these strains grown in decarboxylase media. This study confirms that the ability of *O. oeni* to produce histamine is not a constant characteristic of this species, and it seems to be strain dependent. This opposing results are probably due to the different microbial population present in grapes and wineries from different geographical regions and countries.

To date, there has not been any report on the role of *Leuconostoc* strains in the formation of biogenic amines in wine. In our study, a high potential to produce tyramine was found in *Leuconostoc mesenteroides* strains. Among the 78 strains isolated from must grape or wine, 3 *Leuconostoc* from a total of 17 were tyramine producers (Table 2). As shown in Table 1, two of them (BIFI–61 and BIFI–70) were isolated from wines and BIFI–60 from must. Previously, González de Llano et al. (1998) described two

strains of *Leuconostoc* from dairy origin showing tyrosine decarboxylase activity. These results suggest that *Leuconostoc* may be responsible for tyramine production in wines, in addition to *L. brevis* strains previously described (Moreno–Arribas and Lonvaud–Funel, 1999; Moreno–Arribas et al., 2000).

Several of the biogenic amine–forming species are of importance in food fermentations. In cheese, the role of contaminating strains of *L. brevis* and *L. buchneri* in the formation of tyramine and histamine has been established clearly (Joosten and Northolt, 1989). It can be derived from investigations of Straub et al. (1995) that some strains of *L. buchneri* may also contribute to the formation of putrescine and cadaverine. In the present study, the formation of putrescine was associated with two strains of *L. buchneri* (Table 2). In both strains putrescine was originated from ornithine decarboxylation.

Putrescine, the biosynthetic precursor of polyamines, is produced in *E.coli* by either of two pathways (Morris and Jorstad, 1970). Pathway I involves the decarboxylation of ornithine; pathway II involves the decarboxylation of arginine to agmatine, followed by the removal of urea from agmatine by agmatine ureohydrolase. Both decarboxylases are known in two forms: biosynthetic and degradative. Biosynthetic (or constitutive) decarboxylases are produced when bacteria are grown at neutral pH in minimal medium, and biodegradative (or induced) decarboxylases, which can be induced to high levels, are produced when cells are grown in an acidic, enriched medium containing the corresponding amino acid precursor (Tabor and Tabor, 1985). Considering that arginine is quantitatively one of the most important amino acids in grape musts and wines (Sponholz, 1991; Spayd and Andersen–Bagge, 1996; Moreno–Arribas et al., 1998), we checked the production of putrescine from arginine by the arginine decarboxylase pathway. A high number of strains, mainly oenococci, showed a

purple color in liquid decarboxylase culture indicating the presence of a substance able to alkalinize the media. However when these cultures were analyzed for the presence of biogenic amines by HPLC, none of them showed putrescine or agmatine production. As mentioned above, some reports have described false-positive reactions in some of these media due to the formation of different alkaline compounds (Rodríguez-Jerez et al., 1994; Roig-Sagués et al., 1996). Some wine LAB are known to degrade L-arginine via the arginine deiminase pathway producing ornithine, ammonia, carbon dioxide, and ATP (Granchi et al., 1998; Tonon et al., 2001; Mira de Orduña et al., 2001; Arena et al., 2002). These generated compounds may be responsible for the alkalization observed, giving false-positive reactions in the decarboxylase media supplemented with arginine.

Several biogenic amines-forming species are of importance in winemaking process (Coton et al., 1998; Moreno-Arribas et al., 2000). Some of the positive strains might be used as malolactic starter without knowledge of their potential to form biogenic amines. Therefore, the inability to form these compounds needs also to be confirmed for the microorganisms generally regarded as safe. We have isolated the functional oenoccoci from commercial starters and examined these strains for *in vitro* amine production. None of the commercial malolactic starters tested was able to produce histamine, tyramine and putrescine.

The results of biogenic amine production in a synthetic medium confirm that the capability to produce amines might be strain dependent rather than being related to specific species. However in our screening, this property seems to be more common among strains of particular species, e. g., *Leuconostoc* and tyramine production (Table 2).

In summary, the decarboxylase screening medium showed several advantages: simplicity, easy recognition of the purple color, and good correlation with the chromatographic analysis. However, this screening medium failed to detect putrescine and agmatine production from arginine decarboxylation due to the high number of false positive results, so that these compounds could only be determined by HPLC. Using this media, we found a low incidence of LAB strains able to produce biogenic amines. These results for biogenic amine production in laboratory media does not imply similar behavior in a food product. It should be considered that wines are complex systems with a wide number of factors influencing microbial growth and decarboxylase activity.

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Table 1

Origin of the LAB strains studied

Strain number	Species	Source
30a (ATCC 33222)	*Lactobacillus* sp.	ATCC
CECT 5354 (ATCC 367)	*Lactobacillus brevis*	CECT
BIFI–32, BIFI–33, BIFI–65, BIFI–68, BIFI–75, BIFI–76, BIFI–77, BIFI–78	*Lactobacillus buchneri*	Wine
BIFI–37, BIFI–64	*Lactobacillus fructivorans*	Wine
BIFI–42	*Lactobacillus hilgardii*	Grape must
BIFI–31, BIFI–34, BIFI–35, BIFI–38, BIFI–39, BIFI–40, BIFI–41, BIFI–71, BIFI–72, BIFI–73	*Lactobacillus plantarum*	Wine
BIFI–36, BIFI–62, BIFI–63, BIFI–66, BIFI–79	*Lactobacillus* sp.	Wine
BIFI–43, BIFI–44, BIFI–45, BIFI–47, BIFI–48, BIFI–49, BIFI–50, BIFI–51, BIFI–52, BIFI–57, BIFI–60	*Leuconostoc mesenteroides*	Grape must
BIFI–53, BIFI–54, BIFI–55, BIFI–61, BIFI–70, BIFI–74	*Leuconostoc mesenteroides*	Wine
CECT 4100 (ATCC 23279) (DSM 20252)	*Oenococcus oeni*	CECT
Uvaferm ALPHA, Uvaferm MLD	*Oenococcus oeni*	Lallemand
Viniflora OENOS, Viniflora CH35	*Oenococcus oeni*	Christian Hansen
BIFI–46	*Oenococcus oeni*	Grape must
BIFI–1, BIFI–2, BIFI–3, BIFI–4, BIFI–5, BIFI–6, BIFI–7, BIFI–8, BIFI–9, BIFI–10, BIFI–11, BIFI–12, BIFI–13, BIFI–14, BIFI–15, BIFI–16, BIFI–17, BIFI–18, BIFI–19, BIFI–20, BIFI–21, BIFI–22, BIFI–23, BIFI–24, BIFI–25, BIFI–26, BIFI–27, BIFI–28, BIFI–29, BIFI–69, BIFI–80, BIFI–81, BIFI–82	*Oenococcus oeni*	Wine
BIFI–67	*Pediococcus* sp.	Wine

The CECT strains were kindly provided by Dr. F. Uruburu.
O. oeni commercial strains were kindly submitted by A.Palacios (Lallemand) and A. Lund–Nielsen (Christian Hansen)
Abbreviations: CECT, Colección Española de Cultivos Tipo; ATCC, American Type Culture Collection; DSM, Deustche Sammlung von Mikroorganismen und Zellkulturen; BIFI, Colección de bacterias del Instituto de Fermentaciones Industriales.

Table 2

Biogenic amine production by lactic acid bacteria from Table 1 in modified decarboxylase media supplemented with histidine, tyrosine, ornithine or arginine

		Histamine		Tyramine		Putrescine			
		His		Tyr		Orn		Arg	
Lactic acid bacteria	N[a]	MD[b]	HPLC[c]	MD	HPLC	MD	HPLC	MD	HPLC
L. brevis ATCC 367[d]		0	ND[e]	1	1 (0.4 gl^{-1})	1	ND	1	ND
Lactobacillus 30a[d]		1	1 (1.3 gl^{-1})	0	ND	1	1 (0.9 gl^{-1})	1	ND
O. oeni DSM 20252[d]		0	ND	0	ND	0	ND	0	ND
Lactobacillus buchneri	8	0	ND	0	ND	2	2 (0.9 gl^{-1})	8	ND
Lactobacillus fructivorans	2	0	ND	0	ND	0	ND	1	ND
Lactobacillus hilgardii	1	0	ND	0	ND	0	ND	0	ND
Lactobacillus plantarum	10	0	ND	0	ND	0	ND	2	ND
Lactobacillus sp.	5	0	ND	0	ND	0	ND	4	ND
Leuconostoc mesenteroides	17	0	ND	3	3 (0.8-1.1 gl^{-1})	0	ND	4	ND
Oenococcus oeni	38	0	ND	3	ND	0	ND	33	ND
Pediococcus sp.	1	0	ND	0	ND	0	ND	1	ND

[a]N, number of strains analyzed.
[b]MD, number of positive strains in modified decarboxylase media.
[c]HPLC, number of positive strains by RP-HPLC and concentration range of biogenic amine produced.
[d] control LAB strains.
[e]ND, Not detected ($<2 \times 10^{-5}$ gl^{-1}).