Ann Microbiol (2011) 61:173–178
DOI 10.1007/s13213-010-0073-9

ORIGINAL PAPER

Chemical and molecular methods for the control of biogenic amine production by microorganisms

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Received: 8 February 2010 / Accepted: 27 April 2010 / Published online: 16 June 2010

Abstract The objective of this study was to design and develop an analytical protocol using chemical and molecular techniques to detect microorganisms able to produce biogenic amines (BAs). The protocol consists of: an initial screening by TLC (Thin Layer Chromatography) to detect BA production by microorganisms; multiplex PCR to identify decarboxylase genes in the BA-positive samples; and HPLC to quantify BA production. The protocol was used to verify the ability of a *Lactobacillus hilgardii* strain isolated from wine to produce histamine during malolactic fermentation. The results showed that this analytical approach is an useful tool to determine the production of BAs by microorganisms. Commercial dry yeast starters routinely used in wine-making were examined by this protocol and the results showed that the presence of bacterial contamination in these preparations is a possible origin of BAs in wine.

Keywords Biogenic amines (BAs) · Thin layer chromatography (TLC) · HPLC · PCR · Lactic acid bacteria

Introduction

Wine, like other fermented foods, can contain biogenic amines (BAs) (Buteau and Diutshaever 1984; Baucom et al. 1986), and the main BAs in wine are histamine, putrescine, and tyramine (Coton et al. 1998). BAs are formed by decarboxylation of free amino acids by specific enzymes, including histidine decarboxylase (*hdc*) for histamine, tyrosine decarboxylase (*tdc*) for tyramine, and ornithine decarboxylase (*odc*) for putrescine.

Some research groups support the view that BAs are formed in wine-making mainly during malolactic fermentation (Vidal-Carou et al. 1990). According to several authors, the formation of BAs might be due to spoilage bacteria before, during, or after food processing and it is associated with poor food hygiene and inefficient technology (Zee et al. 1983; Vidal-Carou et al. 1991; Halasz et al. 1994; Bover-Cid et al. 2003).

Both qualitative and quantitative methods to identify and measure the production of BAs by microorganisms have been described. Many microbiological screening procedures involve the use of a differential medium containing a pH indicator (Bover-Cid and Holzapfel 1999).

The TLC method (Garcia-Moruno et al. 2005) is a simple and rapid solution to the false-positive reactions reported in routine screening procedures.

PCR technique, using specific primers, can detect the presence of the *hdc*, *odc* and *tdc* genes that code for the decarboxylase enzymes (Le Jeune et al. 1995; Coton et al. 1998; Lucas and Lonvaud-Funel 2002; Marcobal et al. 2004; Costantini et al. 2006). Assays can be done as single reactions or in multiplex (simultaneous amplification of all the genes in the same PCR reaction) (Coton and Coton 2005; Marcobal et al. 2005; de Las Rivas et al. 2006).

The quantification of BAs is generally accomplished by HPLC (Busto et al. 1997; Mafra et al. 1999; Vidal-Carou et al. 2003). This technique allows the acquisition of reliable quantitative data and good resolution of various amines. The disadvantages are the time-consuming preparation of

This paper is part of the special issue "Wine microbiology and safety: From the vineyard to the bottle (Microsafetywine)", 19–20 November, 2009, Martina Franca (Italy).

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the sample and the requirement of costly, sophisticated equipment and specially trained staff.

Here, we applied an analytical protocol that involves the use of TLC, PCR-multiplex, and HPLC techniques for screening lactic acid bacteria producing BAs. The protocol was used to determine whether contaminating bacteria in commercial starters have the ability to produce the BAs histamine, putrescine, and tyramine, and whether this ability is maintained under wine-making conditions at laboratory scale.

In this work, the protocol was used to verify the biosynthesis of histamine in wine by a strain of *Lactobacillus hilgardii* that had been identified by TLC and PCR as a histamine producer.

Materials and methods

Microorganisms and growth conditions

A strain of *L. hilgardii*, isolated from wine, able to produce histamine was obtained from the collection of CRA-Centro di Ricerca per l'Enologia (ISE 5211). Commercial selected strains of bacteria (16 *Oenococcus oeni*) and yeasts (14 *Saccharomyces cerevisiae*) used in wine-making as starter cultures for malolactic and alcoholic fermentation were obtained from different manufacturers.

The commercial bacterial strains were grown at 30°C in de Man Rogosa Sharpe (MRS) broth; *Oenococcus* at pH 4.8 and *Lactobacillus* at pH 6.

Commercial strains of dry yeast were rehydrated for 30 min at 40°C in 5% sucrose and then grown at 25°C in YEPG (1% yeast extract, 1% peptone, 2% glucose).

The solid medium used for isolation of bacteria was MRS-agar (2% (w/v) agar).

Fermentation

Two commercial yeast preparations were tested for their ability to produce BAs during alcoholic fermentation of grape juice. *S. cerevisiae* commercial starters A and D were tested to determine the formation of BAs in wine. Fermentation took place in a 300-mL flask with 200 mL of pasteurized grape juice obtained from red grapes (Biotta, Tagerwilen, Switzerland) which had a pH of 3.2 and contained 172 g/L of sugars. The trials were done in duplicate. At the end of the fermentation, the wines were analyzed by HPLC to detect and quantify any BA present.

Trials of malolactic fermentation were done with two different Italian wines (Sangiovese and Cesanese d'Affile) using *L. hilgardii* ISE 5211 (10^6 cell/mL) known to be able to produce histamine. Fermentations were done in duplicate at 20°C in 100 mL of wine. The characteristics of the two wines used are given in Table 1. At the end of fermentation, the wines were analyzed by HPLC to detect and quantify any BA present.

TLC method

The TLC assays were done as described by Garcia-Moruno et al. (2005).

Oenococcus oeni strains were grown in MRS broth and yeasts were grown in YEPG broth as described above. The broths were supplemented with the BA precursor amino acids histidine, ornithine, and tyrosine (each at 5 mg/mL), and samples were taken after 3, 6, 9, and 12 days of growth.

Amines were converted to their fluorescent dansyl derivatives, fractionated on silica gel plates (silica gel 60 F254s; Merck) using chloroform/triethylamine (4:1, v/v) as the mobile phase, and the spots were visualized under UV light.

Multiplex PCR method

DNA extraction Total bacterial DNA was extracted as described (Arena et al. 2002). The dried pellet was suspended in an appropriate volume of TE solution (10 mM Tris–HCl, 1 mM EDTA) and stored at −20°C.

PCR amplification PCR amplification was done in a multiplex assay to detect the *hdc*, *tdc* and *odc* genes, simultaneously. The PCR mixture (20 µl) was 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate, 0.5 U *Taq* DNA polymerase (Sigma, St. Louis, MO), 0.4 mM primers for *tdc* and *hdc* and 1 mM primer for *odc* (MWG, Ebersberg, Germany) as described (Marcobal et al. 2004; Costantini et al. 2006).

The 30 cycles of PCR were done with an I-cycler (Bio-Rad Laboratories, Richmond, CA) with the following steps: 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. The amplification products were subjected to electrophoresis in 1.2% (w/v) agarose gel (Sigma) in Tris–acetate buffer (TAE), stained with ethidium bromide

Table 1 Characteristics of Cesanese d'Affile and Sangiovese wines before *L. hilgardii* ISE 5211 inoculation

	Histidine (mg/L)	Histamine (mg/L)	Malic acid (g/L)	Lactic acid (g/L)
Sangiovese	3.00±0.28	0	2.50±0.05	0.22±0.06
Cesanese d'Affile	5.00±0.36	0	3.45±0.09	0.26±0.05

and visualized under UV light (GelDoc 2000; Bio-Rad). Single assays were done when a particular gene had to be verified.

To taxonomically identify the bacterial species, the 16S rDNA gene was amplified by PCR using primers as described (Marchesi et al. 1998). The PCR product was sequenced by MWG. Sequence similarity searches were done with the BLAST analysis tool obtained from the National Center for Biotechnology (http://www.ncbi.nlm.nih.gov/BLAST/) and using the database Ribosomal Database Project (Cole et al. 2009).

HPLC method

Tyramine, histamine, putrescine, and their precursor amino acids were determined by HPLC as described (Costantini et al. 2009). The fluorescence wavelengths were 340 nm for excitation and 450 nm for emission.

Quantification of the BAs used an internal standard of 15 mg/L norvaline. The BA standard was made in 75% (v/v) methanol and contained 20 mg/L each of histamine, putrescine, and tyramine (all purchased from Sigma). The amino acid standard was 20 mg/L each of histidine, ornithine, and tyrosine in 75% (v/v) methanol. The detection limits were 0.1 mg/L for the amines and 0.05 mg/L for the amino acids.

Malolactic fermentation was monitored by HPLC as described (Cane 1990). The detection limit for malic acid and lactic acid was 0.1 g/L.

Results and discussion

In the analytical protocol described here, microorganisms were screened by TLC. The bacteria found to be amine producers were subjected to multiplex PCR analysis to identify the decarboxylase genes, as described in "Materials and methods". In the last part of the protocol, the bacteria were tested for the ability to produce BAs under wine conditions, i.e., during alcoholic and/or malolactic fermentation, by quantification of these compounds with HPLC.

TLC screening

The commercial preparations of bacteria and yeast starters were screened by TLC.

In the analysis of bacterial starters, none of the commercial *O. oeni* produced the BAs in study (results not shown). These results agree with Straub et al. (1995) and Moreno-Arribas et al. (2003), who did not find commercial strains of *O. oeni* that were able to produce histamine, tyramine, or putrescine.

Table 2 Biogenic amine production by commercial yeast preparations

Yeast starter (*S. cerevisiae*)	Amine
A	Tyramine
B	—
C	Tyramine
D	Tyramine, histamine
E	—
F	—
G	—
H	—
I	—
L	—
M	—
N	—
P	—
Q	Tyramine, histamine

— Not detected

The data obtained from the analysis of yeast starters (Table 2) showed that two commercial preparations were able to produce tyramine and another two could synthesize both histamine and tyramine. These positive preparations were, therefore, further studied to determine if it was the yeast that had this ability or whether the production of BAs was the result of bacterial contamination.

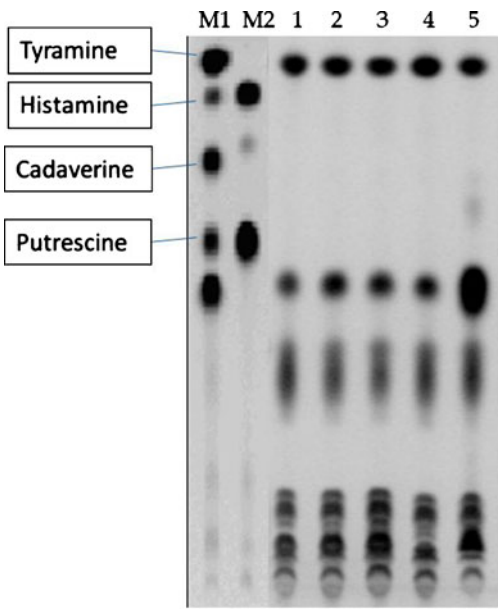

Fig. 1 A TLC plate showing the production of tyramine by contaminating bacteria. *Lane M1* Standard containing tyramine, histamine, cadaverine, and putrescine. *Lane M2* Standard of histamine and putrescine. *Lanes 1–4* Contaminating bacteria isolated from commercial yeast starters. *Lane 5 Lactobacillus brevis* tyramine producer as a positive control

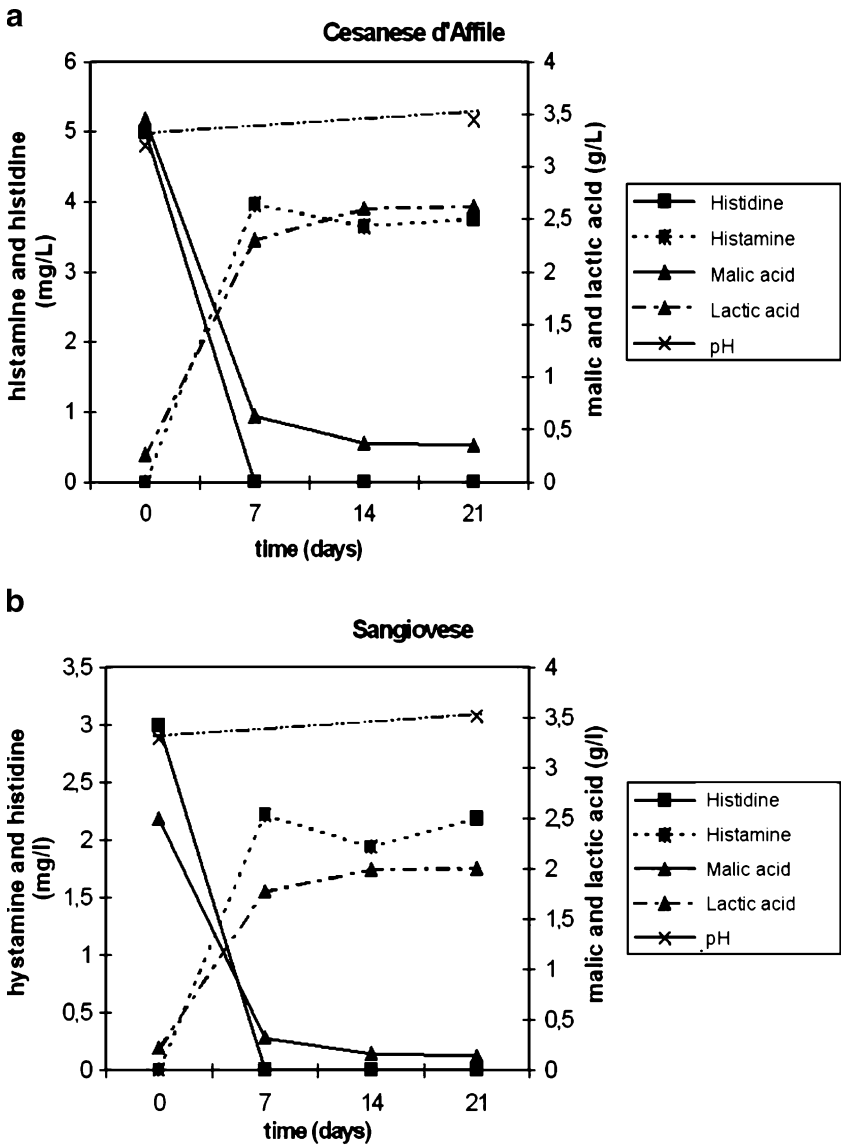

Fig. 2 Trends of malolactic fermentation and histamine production in Cesanese d'Affile (**a**) and Sangiovese (**b**) wines by *Lactobacillus hilgardii* ISE5211

As described (Costantini et al. 2009), rehydrated yeasts were plated on YEPG-agar containing 0.1% (w/v) ampicillin to prevent bacterial growth. After 48 h, single colonies were picked and inoculated into YEPG broth containing the amino acid precursors histidine, ornithine, and tyrosine. Samples were taken after 9 days and analysed by TLC. The results showed that none of the pure yeast cultures was able to produce histamine, tyramine, or putrescine.

Therefore, to check if the amines found by TLC were due to a contamination, 1-ml samples of rehydrated and diluted yeasts were plated on MRS-agar containing 0.1% (w/v) cycloheximide to prevent the growth of yeast and to isolate any bacterial contaminant. Bacterial colonies were isolated and tested by TLC for their ability to produce amines. The results indicated that four commercial yeast starters were contaminated with bacteria that were able to produce amines. Figure 1 shows the tyramine detection by TLC of contaminating bacteria.

PCR detection of amino acid decarboxylase genes

For the positive colonies, the identification of decarboxylase genes was done by PCR multiplex analysis, as noted in "Materials and methods".

The identification of bacterial contaminant species was done by 16S DNA sequencing. Commercial yeast preparations were found to contain lactic acid bacteria, especially *Lactobacillus* spp. and *Pediococcus* spp. The tdc+colonies belong to the species *Lactobacillus brevis* and the hdc+colonies belong to the species *Lactobacillus parabuchneri* and (presumptive) *Lactobacillus rossiae* (95% identity with a *Lactobacillus rossiae*-type strain;

Costantini et al. 2009). Analyzing the sequences with Ribosomal Database Project resulted that they belong to the genus *Lactobacillus*.

PCR analysis confirmed the data obtained by TLC (data not shown).

Alcoholic fermentation and HPLC quantification of BAs

The wines obtained by alcoholic fermentation with contaminated starters A and D were analyzed by HPLC. The data obtained demonstrated that the *L. brevis* strains contained in these preparations were able to produce tyramine in laboratory medium and also in wine. In the must, the initial concentration of the precursor tyrosine was 6.48 mg/L; at the end of the alcoholic fermentation, starter A produced 2.90 mg/L of tyramine and starter D produced 2.69 mg/L of tyramine, while tyrosine concentrations were, respectively, 1.34 mg/L and 1.83 mg/L.

Malolactic fermentation and HPLC quantification of BAs

In an earlier work, bacteria isolated from musts and wines were analyzed for their ability to produce BAs. After screening by TLC, only one strain of *L. hilgardii* was found to be a producer of histamine, the most dangerous amine that can be present in wine. PCR analysis using the specific primers for *hdc* gene PHDC1-PHDC2 confirmed that *L. hilgardii* ISE 5211 possesses this gene and therefore could produce histamine (Costantini et al. 2006).

We asked whether the potential production of BA by this strain could occur during wine-making. For this reason, trials of malolactic fermentations were done with the Italian wines Sangiovese and Cesanese d'Affile, which were chosen because after alcoholic fermentation they contained 3 mg/L and 5 mg/L of histidine, respectively, which is the precursor amino acid for histamine.

The strain of *L. hilgardii,* containing the *hdc* gene was tested for the ability to produce histamine in wine and the ability to perform malolactic fermentation. The malic acid consumption and lactic acid production (Fig. 2) determined by HPLC showed that fermentation was complete within 25 days in both wines, demonstrating that this bacterium can achieve this fermentation. The pH measured before and after malolactic fermentation increased from 3.30 to 3.65 in Sangiovese and from 3.2 to 3.45 in Cesanese d'Affile. Moreover, HPLC detection of the biosynthesis of histamine showed that *L. hilgardii* was able to produce histamine in both wines. In Sangiovese, the 3 mg/L of histidine was converted into 2.3 mg/L of histamine and in Cesanese d'Affile, this bacterium produced 3.45 mg/L of histamine (Fig. 2a, b). In particular, about 85% of the total amount of histamine was produced after 7 days in both wines.

Conclusions

The combined use of TLC, HPLC and PCR represents a useful tool to detect the presence of BA-producing microorganisms in wine, to identify the microorganism, and to verify the ability to produce BAs in wine.

The results led to the conclusion that one possible route of entry of contaminating microorganisms able to produce BAs in wine is via the contamination of commercial dry yeast starters with bacteria.

Moreover, the ability to also synthesize these compounds in wine was demonstrated with a trial of alcoholic and malolactic fermentation, demonstrating that these contaminants represent a real risk for BA presence in wine.

Acknowledgements This work was supported, in part, by a grant from Regione Piemonte Det. Dir. no. 4 of 3 October 2007 and was financed, in part, by COLMIA project DM 16101/7301/08. A. Costantini is a recipient of a PhD fellowship from the CRA-Consiglio per la Ricerca e la Sperimentazione in Agricoltura: Decreto Dirigenziale no. 862 on 10 February 2009.

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