RESOLUTION OIV-OENO 449-2012

MOLECULAR BIOLOGY METHODS FOR THE DETECTION OF BIOGENIC AMINE PRODUCING LACTIC ACID BACTERIA IN WINE

THE GENERAL ASSEMBLY

IN VIEW of Article 2, paragraph 2, no. iv of the Agreement of 3 April 2001 establishing the International Organisation of Vine and Wine,

ON PROPOSAL of the group of experts “Microbiology”

DECIDES to adopt the following Molecular methods for the detection of biogenic amine-producing lactic bacteria in wine

MOLECULAR METHODS FOR THE DETECTION OF BIOGENIC AMINE-PRODUCING LACTIC BACTERIA IN WINE

1. OBJECTIVE:

Detection of specific lactic bacteria strains that have coding genes for the enzymes involved in biogenic amine production in wine. By targeting the suitable gene, PCR can be performed either for detecting the presence of the strains (conventional PCR) or for quantifying their population (quantitative PCR, qPCR). Multiplex PCR can be used to detect the presence of several genes.

2. PRINCIPLE

Most biogenic amine (BA) contamination of wine takes place during malolactic fermentation (MLF) due to the presence of lactic bacteria strains the decarboxylases activities of which convert amino acids into biogenic amines. In addition agmatine (produced by arginine decarboxylation) is deaminated to pustrescine Many strains cannot produce BA. Assessing the potential risk of a BA accumulation in wine at an early stage of production will assist in managing the fermentation process better in order to reduce the spoilage. The method consists in detecting microorganisms that have amino acids decarboxylases and agmatine deiminase. The result cannot indicate the final BA concentrations, but the risk of BA spoilage is linked to the presence of the genes in the bacteria population (Lucas et al., 2008).,

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3. DETECTION OF BA-PRODUCING STRAINS:

3.1. DNA extraction

3.1.1. Extraction from bacterial culture:

The DNA is prepared from pure cultures. Cells of 2 mL of culture (preferably exponential phase) are harvested by centrifugation at 13 000g for 15 min. The pellet is then suspended in 600µL TE buffer (Tris-HCl 10mM, EDTA 1mM) containing lysozyme (10mg/mL) and incubated at 37°C for 30min. Then the extraction is continued by using the available kits according to the manufacturer's instructions. However, the extraction can be also carried out with the usual protocol as follows: one volume of phenol-chloroform_isoamyl alcohol (25:24:1) is added and the mixture centrifuged at 13 000g for 15 min. The upper phase is collected and precipated with ethanol 99%. The pellet is dried and resuspended in the TE buffer.

3.1.2. Extraction of DNA from wine samples bacteria for PCR and qPCR:

From wine samples, DNA is extracted according to Lucas et al. (2008). Freeze-dried yeast cells are added to a final population of 107 cells/mL to the wine samples in order to facilitate the recovery of indigenous micro-organisms and of their DNA. Microorganisms of a 10-ml sample of wine are collected by centrifugation at 5,300 x g for 15 min. Once washed with 1 mL of Tris-EDTA buffer (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA), the pellet is suspended in 300 µl of the same buffer and 200 µl of 0.1mm-diameter glass beads added. Cells are broken through vigorous agitation taking care to cool the tube. The cell lysate is mixed with 300 µl of lyse solution and 200 µl of protein precipitation solution then left on ice for 5 min. Cell debris and proteins are precipitated by centrifugation for 3 min at 10,000 x g. A volume of 600 µl of supernatant is mixed with 100 µl of 10% polyvinylpyrrolidone solution and centrifuged for 10 min at 10,000 x g. The supernatant is collected and nucleic acids precipitated in isopropanol. The DNA pellet washed once with 70% ethanol is dried, and dissolved in 20 µl of sterile water.

3.2. Detection of specific BA-producing strains

3.2.1. PCR conditions:

Amplification by PCR is performed in 25-μl reaction mixture containing 12.5 ng of template DNA, 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 2.5 mM MgCl2, 200µM of each

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dNTP, 1 □M of each primer, and 1 U of DNA polymerase. Oligonucleotide primer sequences for the amplification of internal fragments of the genes coding histidine-, tyrosine-, ornithine decarboxylase, and agmatine deiminase by PCR have been designed by a number of research groups Table 1.

The reactions are performed in a PCR System according to the cycling parameters given in Table 2.

Amplified products are analysed by electrophoresis in a 1.5 % agarose gel and revealed under UV after staining with ethidium bromide.

3.2.2. Applications:

3.2.2.1. Histamine-producing strains

The hdcA gene that codes for the enzyme histidine decarboxylase (HDC; EC 4.1.1.22), is detected by amplification. Among histamine-producing strains, some Pediococcus (Landete et al., 2005) and Oenococcus oeni strains have been isolated (Coton et al., 1998a). It is not a general feature of these species which explains why in other studies, strains of biogenic amine-producing O. oeni have not been found (Constantini et al., 2006; Moreno-Arribas and Polo, 2008).

3.2.2.2. Detection of tyramine-producing strains

Tyrosine decarboxylase (TDC, EC 4.1.1.25) is more present in heterofermentative lactobacilli (mainly Lactobacillus hilgardii and Lactobacillus brevis) (Moreno-Arribas et al., 2000). Lucas and Lonvaud-Funel (2002) designed a degenerate primer set for the detection of tdc gene fragments in L. brevis strains. Later, new primers were designed, (Marcobal et al., 2005; Constantini et al., 2006; Lucas et al., 2003).

3.2.2.3. Putrescine-producing strains (via ornithine decarboxylase)

Ornithine decarboxylase (ODC, EC 4.1.1.17) catalyses the conversion of ornithine to putrescine. Marcobal et al., (2004) reported the identification of an ornithine decarboxylase gene (odc) in a putrescine-producing O. oeni strain. Several primer sets are proposed by Marcobal et al., (2005) and Granchi et al., (2006) that specifically detect putrescine-producing O. oeni strains. However, putrescine can also be formed by the deamination of agmatine. Lucas et al., (2007) gives the primer sequences that amplify the corresponding gene.

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3.3. Simultaneous detection of various biogenic amine-producing bacteria by multiplex PCR

3.3.1. PCR conditions:

For multiplex PCR, conditions are the same as those described above but the suitable concentration of the primers needs to be optimized. The reactions are performed in a PCR System using the cycling parameters described in Table 2. Amplified products are analysed by gel electrophoresis as above.

3.3.2. Applications:

Multiplex amplification methods are used to reduce the quantity of reagent, labour costs and time, because several genes are detected simultaneously. This is suitable for the routine screening for BA producing lactic bacteria in wine. Multiplex PCR assays for the detection of decarboxylases in fermented food and beverages, including wine have been described by Constantini et al., (2006), Coton and Coton (2005), De las Rivas et al. (2006) and Marcobal et al. (2005). For instance, Marcobal et al., (2005) selected three pairs of primers for a multiplex PCR assay for the simultaneous detection of lactic bacteria strains which potentially produce histamine, tyramine and putrescine.

4. QUANTIFICATION OF BA- PRODUCING STRAINS IN WINE BY Q-PCR (QUANTITATIVE PCR)

A qRT-PCR method to detect and to quantify the histamine, tyramine and putrescine-forming bacteria in wines is described in Lucas et al. (2008) and Nannelli et al. (2008)..

4.1. DNA extraction:

DNA is extracted from wine samples as described in 3.1.2.

4.2 Conditions for quantitative PCR amplification

The primers are listed in table 3.

Reactions of 20 µl are performed in the reaction mixture containing 10 pmol of each primer, one tenth of the purified DNA from a sample of wine (2 µl of a 20-µl DNA preparation) and 10 µl 2 × SyBr Green Mix with the cycling program: 5 min at 95°C, 40 cycles (30 s at 95°C, 30 s at 55°C and 30 s at 72°C), followed by melting curve analysis. The melting temperature of the amplification products and the cycle threshold (CT) are calculated automatically

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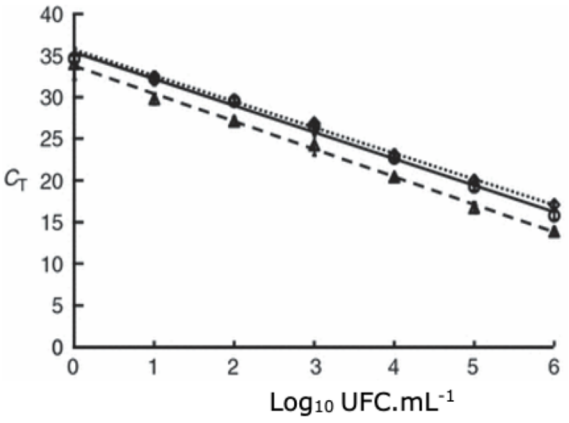


Figure 1 Quantitative PCR standard curves for the detection of tyramine- and putrescine-producing LAB. The templates are DNAs purified from wine samples inoculated with 1–10^6 UFC/ml lactic bacteria with the gene for TDC (diamonds, dotted line), AgDI (circles, plain line) or ODC (triangles, dotted line). Cycle threshold (C_T) values are averages of three replicates. Standard curve equations and coefficients of correlation were,

for TDC: $y = -1{\cdot}3141\ \ \mathrm{Ln}(x) + 35{\cdot}612$, $R^2 = 0{\cdot}997$,

for AgDI: $y = -1{\cdot}3837\ \ \mathrm{Ln}(x) + 35{\cdot}374$, $R^2 = 0{\cdot}995$,

for ODC: $y = -1{\cdot}444\ \ \mathrm{Ln}(x) + 33{\cdot}745$, $R^2 = 0{\cdot}999$

TDC = tyrosine decarboxylase
AgDI = agmatine deiminase
ODC = ornithine decarboxylase

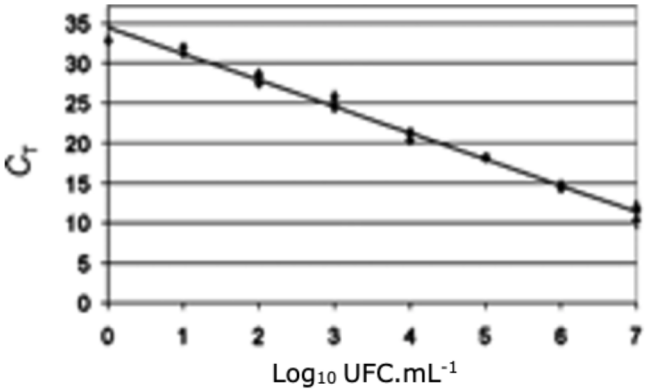


Figure 2 Quantitative PCR standard curve obtained with DNA of HDC^+ lactic bacteria cells serially diluted in sterile wine with yeasts added. C_T values are averages of results from three replicates. The coefficient of correlation (R2) was 0.996.

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Table 1 Oligonucleotide primers for the detection of biogenic amine producing wine lactic bacteria.

Primer name	Primer 5′□3′sequence	Reference
Primers designed for the detection of histamine producing wine LAB		
HDC3	GATGGTATTGTTTCKTATGA	Coton and Coton (2005)
HDC4	CAAACACCAGCATCTTC	Coton and Coton (2005)
Primers designed for the detection of tyramine producing wine LAB		
41	CAYGTNGAYGCNGCNTAYGGNGG	Marcobal *et al.* (2005)
42	AYRTANCCCATYTTRTGNGGRTC	Marcobal *et al.* (2005)
TD5	CAAATGGAAGAAGAAGTAGG	Coton *et al.* (2004)
TD2	ACATAGTCAACCATRTTGAA	Coton *et al.* (2004)
Primers designed for the detection of putrescine producing wine LAB		
4	ATNGARTTNAGTTCRCAYTTYTCNGG	Marcobal *et al.* (2005)
15	GGTAYTGTTYGAYCGGAAWAAWCAYAA	Marcobal *et al.* (2005)
OdF	CATCAAGGTGGACAATATTTCCG	Granchi *et al.* (2006)
OdR	CCGTTCAACAACTTGTTTGGCA	Granchi *et al.* (2006)
AGDIfor	GAACGACTAGCAGCTAGTTAT	Lucas *et al.* (2007)
AGDIrev	CCAATAGCCGATACTACCTTG	Lucas *et al.* (2007)

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K=G or T; R=A or G; W=A or T; Y=C or T; S=C or G; M=A or C; D=A, G, or T; N=A, G, C, or T.

Table 2 PCR conditions used to detect biogenic amine-producing wine lactic bacteria

Gene	Primer set	Amplicon Size (pb)	PCR Step 1	PCR Step 2	PCR Step 3	Cycle number	Reference
hdc	HDC3/HDC4	440	95 °C, 30s	52 °C, 30s	72 °C, 2 min	35	Coton and Coton (2005)
tdc	41/42	213	95 °C, 30s	52 °C, 30s	72 °C, 2 min	30	Marcobal et al., (2005)
	TD5/TD2	1133	95 °C, 30s	52 °C, 30s	72 °C, 2 min	35	Coton et al. (2004)
odc	4/15	972	95 °C, 30 s	52 °C, 30s	72 °C, 2 min	30	Marcobal et al., (2005)
	OdF/OdR	500	95°C, 30s	54°C,30s	72°C,2min	27	Granchi et al., (2006)
agdI	AGDIfor/AGDIrev	542	95 °C, 30s	55 °C, 30s	72 °C, 2 min	35	Lucas et al. 2007

Table 3: Sequences of the primers used in qPCR for quantification of histamine, tyramine and putrescine-producing bacteria in wines. (Lucas et al, 2008; Nannelli et al.,2008))

Gene	Primer name	Sequence 5'□3'	Length amplicon

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Histidine decarboxylase	hdcAf	5'-ATGAAGCCAGGACAAGTTGG	84 bp
	hdcAr	5'-AATTGAGCCACCTGGAATTG	
Tyrosine decarboxylase	tdcf	5'-CAAATGGAAGAAGAAGTTGG	103bp
	tdcr	5'-GAACCATCAGCA ACAATGTG	
Agmatine Dihydrolase (Deiminase)	agdif	5'-ATGCCCGGTGAATTTGAA	90bp
	agdir	5'-TTGCGC TGGTTTAGCACC	
Ornithine decarboxylase	odcf	5'-TGCA CTTCCATATCCTCCAG	127bp
	odcr	5'-GAATTTCTGGAGCAAATC CA	

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