Journal of Chromatography A, 1218 (2011) 7550–7556

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Fast and sensitive detection of genetically modified yeasts in wine

Carlos León [a], Virginia García-Cañas [a], Ramón González [b], Pilar Morales [b], Alejandro Cifuentes [a,*]

[a] Laboratory of Foodomics, Institute of Food Science Research (CSIC), Nicolas Cabrera 9, Campus de Cantoblanco, 28049 Madrid, Spain
[b] Instituto de Ciencias de la Vid y del Vino (CSIC-UR-CAR), Madre de Dios 51, 26006 Logroño, Spain

ARTICLE INFO

Article history:
Available online 22 January 2011

Keywords:
Transgenic yeast
Multiplex PCR
Capillary gel electrophoresis
Genetically modified organisms
Wine

ABSTRACT

In this work, a novel screening methodology based on the combined use of multiplex polymerase chain reaction (PCR) and capillary gel electrophoresis with laser induced fluorescence (CGE-LIF) is developed for the fast and sensitive detection of genetically modified yeasts in wine. As model, a recombinant EKD-13 *Saccaromyces cerevisiae* strain was selected and different wines were prepared using either recombinant or conventional yeasts. Special emphasis is put on the yeast DNA extraction step, exploring different commercial and non-commercial methods, in order to overcome the important difficulty of obtaining amplifiable DNA from wine samples. To unequivocally detect the transgenic yeast, two specific segments of the transgenic construction were amplified. In addition, a third primer pair was used as amplification control to confirm the quality of the yeast DNA obtained from the extraction step. CGE-LIF provides high sensitivity, good analysis speed and impressive resolution of DNA fragments, making this technique very convenient to optimize multiplex PCR parameters and to analyze the amplified DNA fragments. Thus, the CGE-LIF method provided %RSD values for DNA migration times lower than 0.82% ($n = 10$) with the same capillary and lower than 1.92% ($n = 15$) with three different capillaries, allowing the adequate size determination of the PCR products with an error lower than 4% compared to the theoretically expected. The whole method developed in this work requires less than one working day and grants the sensitive detection of transgenic yeasts in wine samples.

1. Introduction

The adoption of DNA recombinant technology has been considered the fastest growing trend in the history of agriculture, and, over recent years, the full potential of this modern biotechnology has been exploited for its application in modern plant breeding [1,2]. In addition to genetically modified (GM) crops, yeasts and lactic acid bacteria with a long history of use for food production have been subjected to genetic modification by genetic engineering mainly for improving the industrial processing or the quality of the final product [3–5]. The development of transgenic yeast strains using recombinant DNA technology has been the most recent step used by microbiologists to improve specific properties of wine [5]. Recently, two transgenic *Saccharomyces cerevisiae* strains have been commercialized in United States and Canada [6,7] to avoid the need of bacterial malolactic fermentation and the associated risk of bacterial spoilage in case of uncontrolled process (ML01 strain [8,9]) and to reduce ethyl carbamate content (ECMo01 strain [10]). Other genetic modifications in wine strains have been directed to: (i) reduce ethanol production [11–13]; (ii) release volatile aroma aglycones from grape glycosylated precursors [14–16]; (iii) improve the production of desirable volatile esters [17], the chemical stability of wine [18], and the yeast autolysis during the second fermentation of sparkling wines [19].

The development and use of genetically modified organisms (GMOs) for food applications are issues of intense debate and public concern that have pushed the European Union and other countries to establish strict regulations concerning different aspects of GMOs, including risk assessment, marketing, labeling and traceability. To verify the application of such regulations, it is necessary to develop analytical methodologies that can effectively detect GMOs in the food chain. In general, analytical procedures for GMO screening in food are based on DNA amplification by the polymerase chain reaction (PCR) technique. The ability of PCR to amplify specific DNA sequences in a complex DNA extract will depend on the integrity, quantity and purity of the DNA extract. These limiting factors define the amplificability of target DNA sequences by PCR-based methods, and are considered critical issues for GMO analysis in highly processed and complex food samples (chocolate, biscuits, etc.). In the case of wine samples, the presence of tannins, polyphenols and polysaccharides may interfere in DNA extraction and/or inhibit the amplification of isolated DNA [20–25]. In addition to the inhibitory effect of wine matrix on PCR, low quantity of DNA and degradation caused, e.g., by biochemical and enzymatic action during fermenta-

* Corresponding author. Tel.: +34 91 5618806; fax: +34 91 5644853.
E-mail addresses: a.cifuentes@csic.es, a.cifuentes@csic.es (A. Cifuentes).

0021-9673/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.01.052

tion and aging, are important constraints for DNA amplification in wine samples [26]. Strategies based on the use of long incubation periods for DNA precipitation or the use of large sample volumes have been applied to extract sufficient DNA from wine samples for subsequent PCR amplification [26–30]. On the other hand, different approaches have been suggested in order to overcome the inhibitory effect of wine matrix on PCR [25,31]. In this regard, attenuation of amplification by phenolic compounds has been addressed by the addition of molecules that act as polyphenols removers (polyvinylpyrrolidone, polyvinylpolypyrrolidone, activated charcoal, etc.) during the DNA extraction step. In other cases, separation methods, such as PVP-agarose gel electrophoresis purification, have been tailored to remove inhibitors from the DNA extracts from difficult samples as soil [24]. Therefore, an efficient extraction procedure is critical for DNA analysis from wine samples.

It has already been demonstrated that multiplex PCR is a suitable methodology for the simultaneous detection of specific targets in GMO-derived materials [32–35]. However, optimization of multiplex PCR is more complex than simplex PCR as the presence of more primer pairs in the reaction system reduces the robustness of the amplification process [32]. In many cases, the optimization and analysis of multiplex PCR reactions requires high sensitivity and resolution. In addition, sensitive analytical methodologies are necessary for the detection of recombinant yeasts in wine, as yeast DNA may be degraded and present in low concentration. In this regard, capillary gel electrophoresis with laser induced fluorescence (CGE-LIF) detection has proven to be a helpful separation technique during the optimization of multiplex PCR methods as well as for the simultaneous analysis of multiple GMOs in food samples [36]. The aim of this work is, therefore, to develop a novel methodology, based on the combined use of optimum DNA extraction, multiplex PCR amplification and CGE-LIF analysis for the fast and sensitive detection of genetically modified yeast strains in wine.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical reagent grade and used as received. Polyvinylpolypyrrolidone (PVPP) was from Applichem (Darmstadt, Germany); phenol was from LabClinics (Madrid, Spain), 2-propanol, chloroform and glucose were purchased to Scharlau (Barcelona, Spain); ethanol and isoamyl alcohol was from Merck (Darmstadt, Germany); and RNAse A was from Roche (Barcelona, Spain). Peptone and yeast extract were purchased from CONDA Pronadisa (Madrid, Spain). AmpliTaq Gold DNA polymerase, including GeneAmp PCR buffer II and, deoxynucleotides, $MgCl_2$, were from Applied Biosystems (Madrid, Spain). Uracil DNA glycosylase was purchased from New England Biolabs (Berverly, MA). Oligonucleotides were purchased from Bonsai Biotechnologies (Alcobendas, Spain). Tris(hydroxymethyl)aminomethano (TRIS) and EDTA were obtained from Sigma (St. Louis, MO, USA); 2-hydroxyethyl cellulose (HEC, MWav 90000) was from Aldrich (Milwaukee, WI, USA); YOPRO-1 was from Molecular Probes (Leiden, The Netherlands). Separation buffer was stored at 4 °C and warmed at room temperature before use. Water was deionized by using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Samples

S. cerevisiae strain EC1118 is a conventional wine yeast strain commercialized by Lallemand Inc. (Montreal, Canada). EKD-13 is a genetically modified strain of *S. cerevisiae* with an improved capacity to release mannoproteins to the media during the fermentation of the must [18]. Both *S. cerevisiae* strains were grown separately in YPD broth (2% glucose, 2% peptone, 1% yeast extract) as negative and positive controls, respectively. 5 mL of YPD culture containing the reference yeasts were incubated for 24 h at 30 °C. Bottled wine, labelled as Petit Verdot, was donated by Laboratorio Agroalimentario de Jerez (Cádiz, Spain). For experimental production of control and recombinant wine samples, precultures were grown in YPD broth. Two types of grape must, including a blend of red wine grapes (musts 1 and 2) and a Graciano monovarietal red wine (must 3) were used for fermentation assays with EC1118 and the recombinant EKD-13 strains. Must were sulphited in origin to 30–50 ppm of potassium metabisulphite. 200 mL of the unclarified musts (musts 1 and 3), or a must clarified by gentle centrifugation (must 2) were inoculated 1% in volume from a preculture in YPD grown for 48 h at 28 °C. All musts were fermented as for white wines (i.e., without maceration of skins and seeds), but the must differed in colour intensity with a deeper colour for the blend of red grapes (musts 1 and 2) than for the Graciano must (must 3). Fermentation was carried out at a controlled temperature of 25 °C in erlemenyer flasks closed with fermentation locks filled with Vaseline oil. Fermentation kinetics was monitored as CO_2 formation as estimated by daily recording loss of weight through the fermentation lock. Wine fermentation was considered complete after 2 days of constant weight. This was confirmed by HPLC analysis of the main fermentation metabolites (glucose, fructose, glycerol and ethanol). No further clarification was performed on the wine samples.

2.3. DNA extraction and quantification

Different DNA extraction protocols (commercial and non-commercial) were evaluated in this work in order to obtain representative and reliable DNA extracts from the different types of samples.

2.3.1. DNA extracts from yeasts

Extractions of DNA from cultured yeasts were performed using a commercial kit (MasterPure™ Yeast DNA Purification Kit from Epicentre Biotechnologies, Madison, WI) following the instructions given by the manufacturer. Briefly, 300 μL of Yeast Cell Lysis solution were added to the pellet, followed by 150 μL of MPC Protein Precipitation Reagent and 0.5 mL of isopropanol. The DNA extract (50 μL) was incubated with RNase (1 μL) at 60 °C for 30 min. After incubation, 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) was added and homogenized. After centrifugation at 14,000 rpm was performed, the upper phase was transferred to another tube and another extraction with chloroform/isoamyl alcohol (24:1, v/v) was done. Then, 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol were added to the upper phase collected. The solution was kept at −20 °C for 2 h. The precipitate was collected by centrifugation at 14000 rpm for 10 min and washed with 70% ethanol. The remaining ethanol was evaporated at room temperature and the pellet was dissolved in 50 μL TE buffer that is composed of 10 mM Tris–HCl and 1 mM EDTA at pH 8.0.

2.3.2. DNA extracts from wines

Several DNA extraction protocols were evaluated to amplify DNA from wine samples. The experiments were done using Petit Verdot as a model wine sample trying to obtain DNA of good quality and yield in order to get a sensitive amplificability of the *mrp2* gene by PCR. Briefly, 15 mL of the wine sample were centrifuged (10,000 rpm for 5 min at 5 °C) to recover the solid parts in suspension. DNA extraction from the solid parts (pellets) was attempted using three different methods (A, B and C, see below). Alternatively, during method optimization, the following procedure was also assayed as sample pre-treatment in order to remove potential inhibitors from the sample. Briefly, the solid part (pellet) was resuspended in 2 mL water and homogenized by pipetting. The