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ORIGINAL PAPER

Use of interdelta polymorphisms of *Saccharomyces cerevisiae* strains to monitor population evolution during wine fermentation

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Abstract The industrial use of starter cultures containing a consortium of different strains from the same species is nowadays seen as a possible strategy to enhance the organoleptic complexity of wines. To assess the relative contribution of each strain to the final product it is essential to quantify population evolution during the wine fermentation process, which requires strain-specific methods to identify and differentiate each strain. In the present study, a molecular method based on analysis of the polymorphisms exhibited by the PCR-amplification of the delta regions of three *Saccharomyces cerevisiae* strains was developed. A set of three pairs of primers (delta1–delta2, delta12–delta2, delta12–delta21) was used for each strain, and analysis of the resulting polymorphism patterns showed that the delta12–delta2 primer pair exhibited the highest resolution and discriminatory power. Thus, this pair of primers was selected to monitor the population evolution of a laboratory-scale wine fermentation performed in synthetic grape juice that was inoculated with similar amounts of each strain. The results showed that all strains grew together during the exponential growth phase (2–3 days) and maintained high cell density values (10^6–10^7 cfu ml^{-1}) throughout the stationary growth phase without significantly changing their relative population proportion, thus indicating that each strain can influence the chemical composition and final flavor of wine, albeit at different levels. This study also showed that PCR-amplification of DNA delta sequences of *S. cerevisiae* strains is a reproducible, strain-specific and simple method that can be used successfully to monitor yeast strain population dynamics during wine fermentations.

Keywords Strain-specific primer · Interdelta polymorphism · Mixed starter culture · Wine fermentation · Yeast population dynamics · Molecular methods

Introduction

The utilisation of selected *Saccharomyces cerevisiae* strains as starter cultures in wine production is nowadays a common and widely used practice. Grape must microflora comprises many *S. cerevisiae* strains and it is well established that each of those strains contribute differently to the final flavour profile of the wine [9, 18]. In order to preserve the typical characteristics of certain wines produced in a given region, many enologists recommend the selection of strains from amongst the local microflora of grape musts [15]. Furthermore, some wineries select indigenous *S. cerevisiae* strains and use them in consortia to inoculate their grape musts. The use of starter cultures containing strains in consortium, displaying desirable and complementary characteristics, requires reliable techniques able to identify and differentiate the selected strains from the remaining microflora during the fermentation process.

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Several molecular methods have been developed to identify and discriminate industrial yeasts at the species and/or subspecies level and some of them have been applied to monitor yeast strains during wine fermentations. Those methodologies include restriction fragment length polymorphisms (RFLP) of genomic and mitochondrial DNA [6, 16, 20], chromosome karyotyping [2, 11, 19, 21], randomly amplified polymorphic DNA (RAPD) [5, 13, 17, 23], amplified fragment length polymorphism (AFLP) [4], application of microsatellites [1, 7, 8, 19] and, recently, single nucleotide polymorphisms (SNPs) [10].

Molecular typing of *S. cerevisiae* strains via the analysis of interdelta polymorphisms was first proposed by Ness et al. [13]. The *S. cerevisiae* genome contains repetitive DNA sequences, such as delta sequences, that are frequently associated with the Ty1 transposon [3]. As demonstrated by different authors [12, 19], the number and location of these elements have a certain intraspecific variability that can be used as a genetic fingerprint to identify *S. cerevisiae* strains. In a survey of molecular methods for typing wine yeast strains, Schuller et al. [19] showed that both microsatellite typing and interdelta analysis have a discriminatory power similar to that of mtDNA restriction analysis and karyotyping. Those authors concluded that PCR amplification of delta sequences is a very convenient method to use in standard control of industrial wine fermentations since it is rapid, reproducible and very sensitive. In addition, it does not require a high investment in equipment and skilled human resources, which can be viewed as the only disadvantages of microsatellite typing [19]. In a recent study, Vaudano and Garcia-Moruno [22] proposed a new microsatellite method based on the analysis of patterns generated by multiplex PCR that does not require sequence analysis and thus also could be considered as a good compromise between discriminatory ability, time required for analysis and cost.

In a Portuguese Winery (Fundação Eugénio de Almeida) a consortium of three *S. cerevisiae* strains (CCMI 885, CCMI 888 and CCMI 890) that were previously isolated from the local microflora, is commonly used to produce regional white wines. Sensorial analyses carried out by trained sensory panels identified a distinct flavour pattern for wines produced with these three strains (un published data). However, there was a lack of a reliable and simple method that could be used to follow yeast population dynamics during wine fermentation inoculated with a mixture of these strains. The aim of the present work was to develop and apply a PCR interdelta-based method to differentiate these three strains, and to follow the population evolution of each strain during a mixed fermentation carried out under enological growth conditions.

Materials and methods

Yeast strains

Saccharomyces cerevisiae strains CCMI 885, CCMI 888 and CCMI 890, previously isolated from the indigenous microflora of grape musts of Alentejo (Portugal), were obtained from the Culture Collection of Industrial Microorganisms (CCMI, INETI, Portugal). After revitalisation, the lyophilised strains were maintained on YEPD agar (2% glucose, 0.3% yeast extract, 0.5% peptone, 0.3% malt extract, 2% agar) slants at 4°C.

Fermentation conditions and sampling

Fermentation was performed in a 2 l flask containing 1.5 l synthetic grape juice (SGJ) with the composition described by Perez-Nevado et al. [14], containing about 200 g l^{-1} glucose and fructose, pH 3.5. Fermentation was carried out at 18°C without aeration and agitation. Growth medium (SGJ) was inoculated with 1% (v/v) of individual inoculums of *S. cerevisiae* strains (CCMI 885, CCMI 888 and CCMI 890) to attain ca. 10^6 colony forming units (cfu) ml^{-1} of each strain. Inoculums were prepared by transferring the biomass from one YEPD-agar slant (pregrown for 48 h at 30°C) into 50 ml YEPD medium (2% glucose, 0.3 g % yeast extract, 0.5% g peptone, 0.3% malt extract) and incubating at 30°C in an orbital shaker (150 rpm) for 16 h.

Daily samples were taken from the fermentation medium and plated, after appropriately dilution, onto YEPD-agar plates for total cell counts and strain population quantification by PCR-amplification. Filtrates from each sample were used to determine glucose, fructose and ethanol concentrations throughout the fermentation process. For strain population quantification, 30 colonies were picked randomly from one YEPD-agar plate containing around 200–300 colonies, and the DNA from each colony was extracted for PCR-amplification reactions.

DNA extraction and purification

In a first step, the technique used was that described by Xufre et al. [23], with slight modifications. Biomass from one YEPD-agar colony was dissolved in 500 µl extraction buffer (0.2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA). After addition of 10 µl RNase (20 mM) and 5 µl proteinase K (20 mg ml^{-1}), the suspension was incubated at 37°C for 1 h. The cells were homogenised by vortexing for 5 min in the presence of 500 µl phenol/chloroform/isoamyl alcohol (50:48:2). Tris-EDTA buffer (50 µl) was added and the aqueous layer was collected after centrifugation. The DNA

was precipitated with 2 volumes of absolute ethanol. After centrifugation at 13,000 *g* for 15 min, the precipitated DNA was dried and resuspended in 150 µl TE buffer (0.1 M Tris, 0.1 M EDTA, pH 8.0). Aliquots of 25 µl were stored at −20°C. The efficiency of this DNA extraction procedure and the quality of the extracted DNA was analysed on electrophoresis agarose (1% w/v) gels (30 min at 90 V) that were stained with ethidium bromide (0.5 µg ml^{-1}) and visualised in a UV transilluminater. The DNA concentration was determined by measuring the absorbance at 260 nm. The DNA extracted by this method was used in the PCR reactions performed to test the discriminatory power of each pair of primers (delta1–delta2, delta12–delta2; delta12–delta21).

Due to the high number of colonies to examine during the fermentation (330 colonies) we tested a simpler and faster method of DNA extraction. The method consisted of the following procedure: biomass from one colony was resuspended in 20 µl sterile water in a closed Eppendorf tube that was placed in a microwave oven (900 W) and heated at full power for 5 min; 1 µl of this suspension was directly added to the PCR mixture and used in the PCR reactions.

DNA amplification and primers

Four primers were used in the present study, delta1 (5′-CAAAATTCACCTATATCT-3′), delta2 (5′-GTGGATTTTTATTCCAAC-3′), delta12 (5′-TCAACAATGGAATCCCAAC-3′) and delta21 (5′-CATCTTAACACCGTATATGA-3′) in different combinations: delta2–delta1; delta12–delta2 and delta12–delta21. Primers delta1 and delta2 are those previously described by Ness et al. [13] and primers delta12 and delta21 were designed by Legras and Karst [12]. All primers were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden).

Amplification reactions were performed on a Bio-Rad iCycler thermal cycler, using the above-mentioned pairs of primers. Reaction mixture (25 µl) was prepared with 1 µl of the extracted DNA (∼10 ng), 1.0 U *Taq* polymerase (Pharmacia), *Taq* buffer (10 mM Tris–HCl, 50 mM KCl, 0.08% Nonidet P-40), 20 pmol of each primer, 0.2 mM of each dNTP and 2.5 mM $MgCl_2$. After initial denaturation (95°C for 4 min), the reaction mixture was cycled 35 times using the following program: 95°C for 30 s, 52°C for 30 s, 72°C for 90 s with a final extension at 72°C for 10 min. The amplification products were separated by electrophoresis on 2% (w/v) agarose gels submitted to 90 V (constant voltage) for 2.5 h in 0.5 TBE buffer. DNA samples were loaded in gels using a loading buffer [glycerol 30% (w/v), bromophenol blue 0.25%, EDTA 20 mM] that sets low molecular weight markers at 150–50 bp. After staining with ethidium bromide (0.5 µg ml^{-1}), DNA bands were visualised using an UV transilluminator and photographed. In each gel, M100 bp (Amersham Biosciences and Fermentas) were used as molecular weight markers.

In a first step, DNA amplification reactions were repeated at least twice for each strain using the different pairs of primers. After analysis of the resulting electrophoretic patterns, the best primers pair (delta12–delta2) was selected to monitor the population evolution of each strain during the vinification process.

Determination of cell density

Yeast growth was analysed by viable cells enumeration using the classical plate count method. Samples, taken aseptically throughout the fermentation, were inoculated onto YEPD-agar plates after appropriate dilution in sterile water. All plates were incubated at 30°C for 2–4 days and viable cell enumeration was carried out after no increase in cfu was observed.

Analytical methods

Ethanol, glucose and fructose concentrations during the fermentation process were determined on cell-free samples (filtration through 0.45 µm Millipore membranes) by HPLC (Merck Hitachi, Darmstadt, Germany), using a Sugar-Pak™ column (Waters, Milford, MA) connected to a RI detector (L-7490, Merck Hitachi). The column was eluted at 90°C with a degassed aqueous mobile phase containing 50 mg l^{-1} CaEDTA, at a flow rate of 0.5 ml min^{-1}. All samples were analysed in duplicate.

Results and discussion

Initially, we evaluated the ability of different pairs of primers, delta1–delta2, delta12–delta2 and delta12–delta21, to differentiate three *S. cerevisiae* strains (CCMI 885, CCMI 888 and CCMI 890). As expected, distinct polymorphic patterns were formed for each strain according to the pair of primers used (Fig. 1). The primer pair delta1–delta2 gave rise to a very low number of fragments per strain, with one common band (ca. 400 bp) for all strains. The low number of amplified fragments when using the delta1–delta2 pair was ascribed to the weak homology exhibited by the primer delta1 towards the whole sequence of the delta region of the *S. cerevisiae* genome [12]. Nevertheless, for the same pair of primers (delta1–delta2) Legras and Karst [12] found six distinct patterns within 8 *S. cerevisiae* strains, while Schuller et al. [19] found ten distinct patterns within 23 strains. Replacement of primer delta1 by primer delta12 in combination with primer delta2 increased the number of fragments achieved for each strain

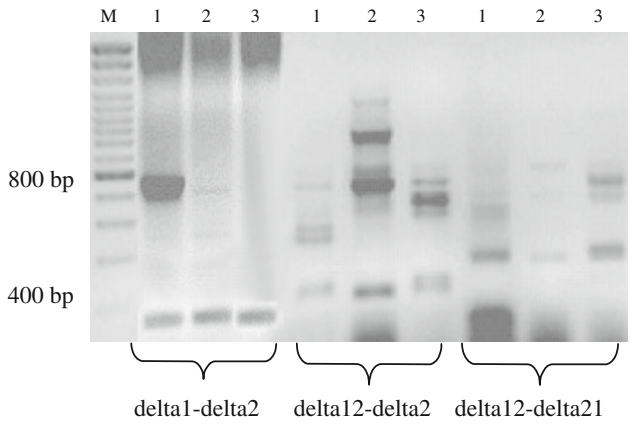

Fig. 1 Molecular typing of *Saccharomyces cerevisiae* strains by interdelta PCR amplification using the pairs of primers indicated. Strains: *1* CCMI 885, *2* CCMI 888, *3* CCMI 890. *M* M100 bp molecular weight marker (Amersham Biosciences and Fermentas)

and gave rise to three distinct patterns. Likewise, Schuller et al. [19] verified that using the pair delta12–delta2 instead of pair delta1–delta2 to type 23 *S. cerevisiae* strains, the number of distinct patterns obtained increased from 10 to 21. The use of the delta12–delta21 pair also resulted in a higher number of amplified fragments in comparison with the delta1–delta2 primer pair. Nonetheless, this pair of primers (delta12–delta21) exhibited a lower discriminatory power than the pair of primers delta12–delta2.

Several DNA samples of each strain, extracted by distinct methods, were used to test the reproducibility of the method. Identical interdelta profiles were obtained for the same strains and the same pair of primers, indicating that this PCR-interdelta method is highly reproducibly (data not shown). These results also showed that direct PCR amplification of DNA extracted from one agar colony using a microwave oven, instead of using the classical phenol–chloroform

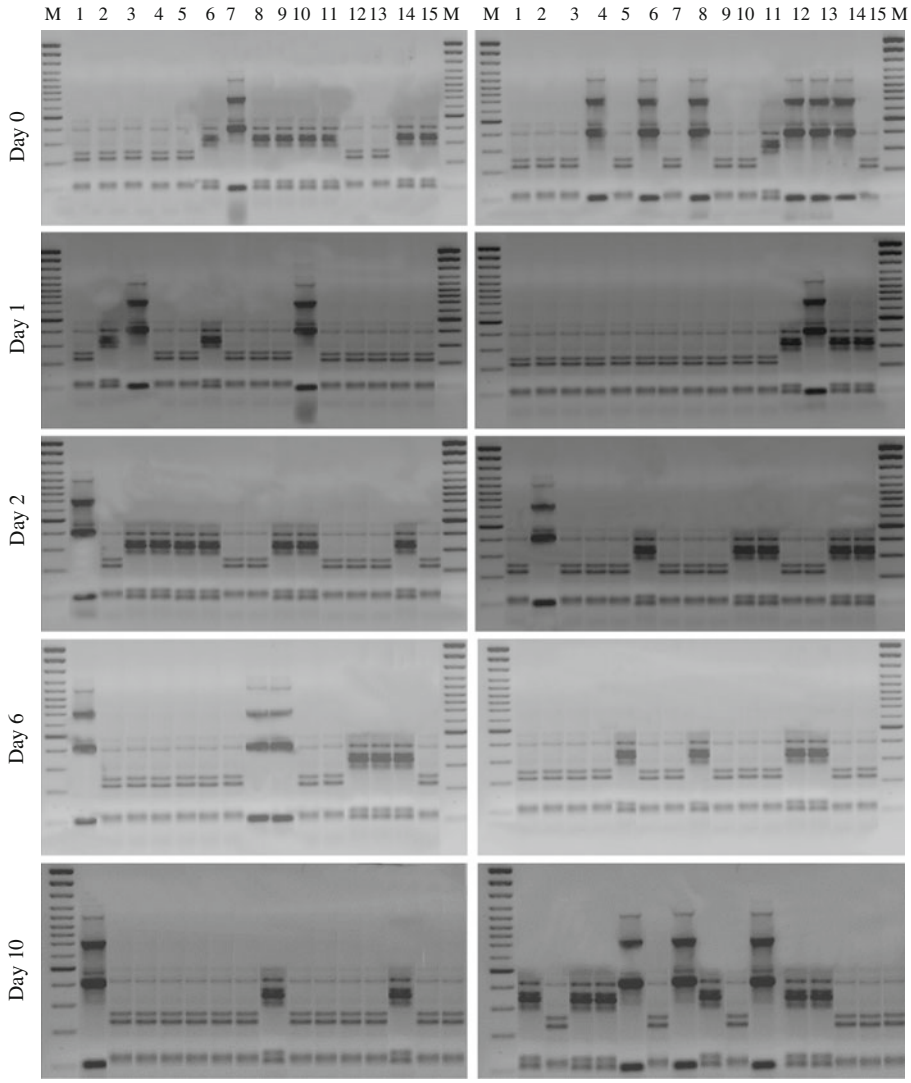

Fig. 2 Electrophoresis profiles obtained by interdelta PCR-amplification with the pair of primers (delta12–delta2) for some of the samples (days 0, 1, 2, 6 and 10) taken from the mixed fermentation inoculated with *S. cerevisiae* strains (CCMI 885; CCMI 888; CCMI 890). For each sample, 30 colonies were analysed. Lane numbers correspond to the different colonies analysed; *M* M100 bp molecular weight marker (Amersham Biosciences and Fermentas)

extraction technique, is a faster and simpler method of DNA extraction that allows reliable results for this PCR-based method. A simple DNA extraction from agar colonies followed by direct PCR procedure was also described by Vaudano and Garcia-Moruno [12].

The primer pair delta12–delta2 was selected and used to monitor yeast population evolution during the course of the mixed fermentation. Fermentation was performed in SGJ and inoculated with ca. 10^6 cfu ml^{-1} of each of three *S. cerevisiae* strains (CCMI 885, CCMI 888, CCMI 890). To quantify the population evolution of each strain during the course of fermentation, samples taken daily (from day 0 to 10) from the fermenting medium, were spread onto agar plates and 30 colonies picked randomly from each plate were analysed by PCR-amplification. Figure 2 represents the electrophoresis profiles obtained for 5 of the total 11 samples analysed. The same strain-specific pattern was revealed for all the colonies analysed (330), thus confirming that this method is highly specific and reproducible.

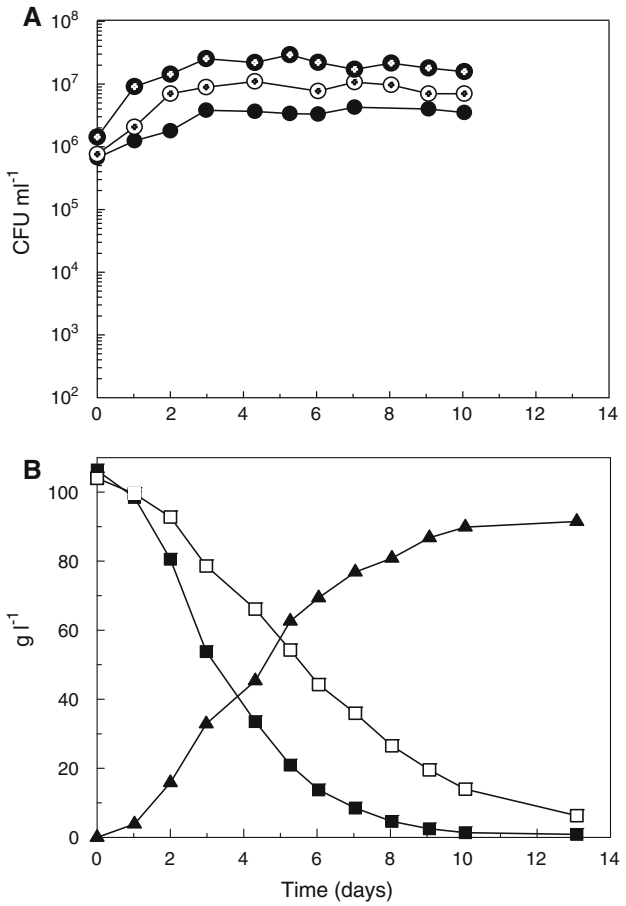

Fig. 3 **a** Microbial growth of *S. cerevisiae* strains CCMI 885 (*white dots on filled circles*), CCMI 888 (*filled circles*) and CCMI 890 (*dark dots on open circles*), and **b** glucose (*filled squares*) and fructose (*open squares*) consumption and ethanol (*filled triangles*) production during the laboratory fermentation performed in synthetic grape juice (SGJ)

Our results also proved that there is no change in the interdelta polymorphisms exhibited during the changing and stressful growth conditions present during the alcoholic fermentation process. Taking the PCR results and the total cfu counts of a given sample together, it was possible to determine the cell density profile of each strain throughout the fermentation process (Fig. 3a). All strains exhibited exponential growth during the first 3 days of fermentation, attaining maximal cell densities of 2.5×10^7 cfu ml^{-1} for strain CCMI 885, 1.1×10^7 cfu ml^{-1} for strain CCMI 890 and 4.5×10^6 cfu ml^{-1} for strain CCMI 888. These cell density values stayed roughly constant throughout the stationary growth phase (from days 3–10). Sugar consumption and ethanol production during the fermentation process are shown in Fig. 3b. Yeast strains consumed glucose faster than fructose, exhausting glucose within 10 days and showing a residual fructose concentration of 6.2 g l^{-1} after 13 days of fermentation. The inability of the yeasts to exhaust all sugars in the media (SGJ) within 13 days might have resulted from the relatively low fermentation temperature (18°C) or from a nutrient (e.g. nitrogen) limitation. A maximal ethanol concentration of 91 g l^{-1} (11.3% v/v) was attained after 10 days, and it remained at this value until the 13th day of fermentation.

Conclusions

The use of interdelta amplification as a strain-typing method allowed the population dynamics of three *S. cerevisiae* strains to be followed during mixed enological fermentation using a simple, rapid and reliable technique of DNA extraction. Analysis of more than 330 colonies by PCR-amplification of delta sequences using the pair of primer delta12–delta2 demonstrated that this method is highly reproducible and strain-specific. With the present technique, it was possible not only to differentiate strains for all samples analysed, but also to assess the relative proportion of each strain during the course of the fermentation process. Although the interdelta PCR-amplification method has been used before to differentiate *S. cerevisiae* strains, its application to monitoring population dynamics during enological fermentations has not yet been reported.

The present study provided scientific evidence that each of the *S. cerevisiae* strains commonly present in consortia used to inoculate grape musts at a Portuguese Winery grow together and maintain high viable cells numbers (above 10^6 cfu ml^{-1}) throughout the fermentation process, thus corroborating the claim of local enologists that the use of this consortium as a starter influences the final flavour of the wine.

Moreover, the present results demonstrate that this PCR-interdelta method is a simple, fast and reliable method to

monitor yeast strains during wine fermentations. With this method it is possible to control the dominance of the starter culture over the indigenous *S. cerevisiae* strains and also to follow the strain population dynamics during wine fermentations. Moreover, by using this PCR procedure together with sensorial and chemical analysis it would be possible to establish correlations between flavour production and strain dominance. Due to its high reproducibility, discriminatory power and good time-requirement/price relationship, this method compares favourably with other techniques such as AFLP, MSP-PCR, RFLPmt, RAPD and PFGE that have also been used to differentiate strains during wine fermentations.

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