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Identification of Wine Yeasts by PCR-RFLP without Previous Isolation on Plate

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Summary

The population of wine yeasts during spontaneous must fermentation was characterized by direct 5.8S-ITS rDNA region amplification without previous plate isolation or enrichment. RFLP analysis was applied to each of the amplification products detected, and the corresponding yeast identifications were made. The method provides a fast and direct way of determining yeast population present during wine fermentation.

Key words: wine yeasts, identification, PCR-RFLP

Introduction

Substantial growth of non-*Saccharomyces* yeasts takes place in the early stages of fermentation and may contribute to some special characteristics of wine (*1*,*2*). The yeasts of the genera *Kloeckera*, *Hanseniaspora*, *Candida*, *Pichia*, and others all grow during the early stages of fermentation. Afterwards, as ethanol concentrations rise, *Saccharomyces cerevisiae* begins to predominate and is responsible for alcoholic fermentation. Identification of the yeasts involved in fermentation process requires plate isolation, enrichment and cultivation of different strains. Nevertheless, in the last few years new methods have been developed to characterize wine yeasts directly without the need for enrichment or isolation (*3*,*4*).

The strains at the species level can be identified using »conventional methods« based on biochemical and physiological characteristics (*5*) or molecular techniques (6). PCR-RFLP has been used to characterize wine yeasts present in the fermentation process after isolation of the yeasts (*7*).

In this study we identified the yeast species present in a sample collected from an industrial wine fermentation vat using PCR amplification and RFLP analysis of the DNA purified directly from the sample, without plate isolation.

Materials and Methods

Sampling

The sample studied was withdrawn from a single vat of white must (capacity: 2×10^5 L) at an early stage of fermentation. Commercial dry yeasts had never been used before as starters at the wine cellar located in the La Mancha region in Spain.

A volume of 100 mL of must was aseptically taken from the fermentation vat and transported to the laboratory in a sterile vessel with glycerol (15 % v/v). The sample was frozen and stored at –40 °C until analysis.

After thawing and homogenization of the must sample, a 12 mL aliquot was centrifuged (4 500 rpm, Hettich model 1617 rotor, 4 °C, 10 min), and the sediment was washed carefully three times in sterile water and then further three times in TE buffer (Tris HCl 10 mM, EDTA 1 mM, pH=8) to yield a final volume of 12

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mL. The washed cells were used for genomic DNA isolation as described by Rose *et al*. (*8*). Both the sterile water and the TE buffer were chilled to 4 °C. 1 ng of genomic DNA was employed as the optimal amount for the amplification reactions.

PCR

The Internal Transcribed Spacers (ITS1 and ITS4) described by White *et al*. (*9*), which amplify the ITS-5.8S region on the rDNA region, were used as primers (MGW, Biotech, Germany). The amplification reaction was performed in a GeneAmp model 2400 PCR System (Perkin-Elmer Corp., Norwalk, Connecticut, USA) under the following conditions: a 50 μL reaction mixture was made up of 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems Division, Foster City, CA, USA), 0.5 μM of each of the primers, ITS1 (19-mer 5′-TCCGT AGGTGAACCTGCGG-3′) and ITS4 (21-mer 5′-TCCTC CGCTTTATTGATATGC-3′), 0.2 mM of each dNTP, 1 × AmpliTaq Gold buffer II, 2.5 mM $MgCl_2$ and 1 ng of template DNA. The mixture was put through an initial denaturing cycle of 10 min at 95 °C, which was followed by 40 cycles comprising 1 min at 95 °C, 2 min at 58 °C, and 3 min at 72 °C, with a final extension step of 10 min at 72 °C (*10*).

The amplification products were separated by electrophoresis in a 1.5 % (w/v) agarose gel with 1 ×TAE using a 100 base pairs (bp) ladder (Biotools, Madrid, Spain) DNA molecular marker.

The different amplification products were purified by isolating each band from the agarose gel using the GENECLEAN II Kit™ (Bio 101, Carlsbad, CA, USA) according to the manufacturer's instructions.

Restriction analysis

To identify the yeasts, the amplified products were treated with two restriction enzymes (*Hinf*I and *Hae*III). In some cases the use of *Dde*I was necessary to discriminate both species. Reaction conditions were created according to the supplier's (Roche Diagnostics, S.L., Barcelona, Spain) instructions. The restriction fragments were checked by electrophoresis in 1.5 % w/v agarose gel plus ethidium bromide (0.5 μg/mL).

Results and Discussion

DNA from the sample was obtained and amplified as indicated in the Materials and Methods section. Six different amplification products with lengths between 400 and 900 bp were discriminated on agarose gel electrophoresis (Fig. 1). Each band was extracted from the gel and carefully isolated using GENECLEAN II ™ for further analysis. The isolation was verified and an enhanced determination of amplification product size was carried out (Fig. 2). Afterwards, each of the amplification products underwent restriction analysis using the enzymes *Hinf*I and *Hae*III separately (Fig. 3).

The size of the different restriction fragments obtained from the six amplification products is shown in Table 1. The identification of the yeasts was achieved by comparing the patterns obtained in this study with those described by Esteve-Zarzoso *et al*. (*11*). *Hansenia-*

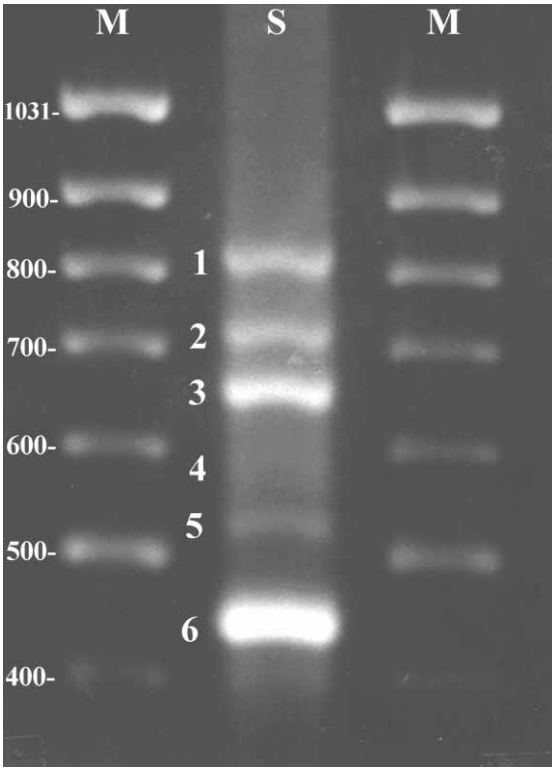

Fig. 1. Agarose gel with PCR products of an amplification of the 5.8S-ITS rDNA obtained without plate isolating. Lanes M: Marker 100 bp (1031, 900, 800, 700, 600, 500, 400, 300, 200, 100, 80). Lane S: Sample of the must

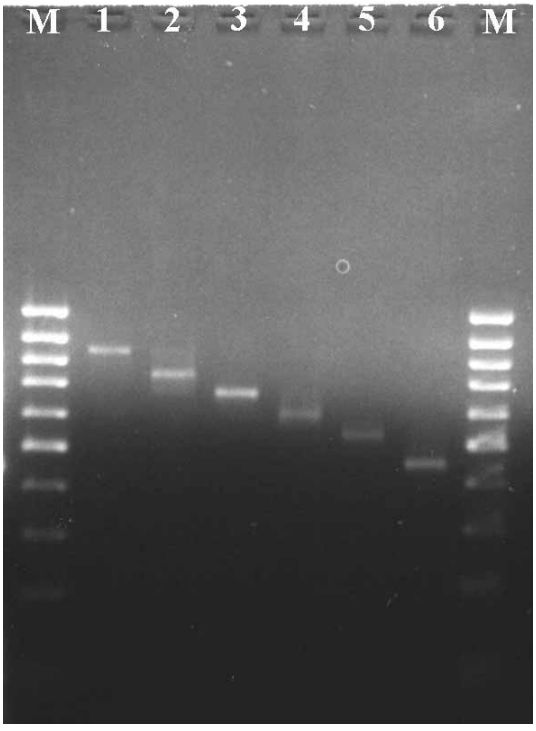

Fig. 2. Agarose gel with PCR products of an amplification of the 5.8S-ITS rDNA obtained after treatment with GeneClean II. Lanes M: Marker 100 bp (1031, 900, 800, 700, 600, 500, 400, 300, 200, 100, 80), Lanes 1–6: Different amplification products (Lane 1: *S.cerevisiae*, Lane 2: *H. uvarum*, Lane 3: *K. thermotolerans*, Lane 4: Not identified, Lane 5: *C. vinaria*, Lane 6: *C. stellata*)

Table 1. Summary of the molecular profiles and their possible identification. Size (bp) of the amplification products (AP) and of the corresponding restriction fragments

Lane	AP	Restriction fragments		Yeast identification
		*Hae*III	*Hinf*I	
1	880	320+230+180+150	365+365+155	*Saccharomyces cerevisiae*
2	750	750	350+200+180	**Hanseniaspora uvarum*
3	700	310+210+95+95	350+345	*Kluyveromyces thermotolerans*
4	650	330+140+85	360+340	Unidentified
5	560	460+100	250+200+100	*Candida vinaria*
6	475	475	240+235	*Candida stelliata*

**Hanseniaspora uvarum* was differentiated from *H. guilliermondii* with the use of *Dde*I (300, 180, 95, 90, 85 bp)

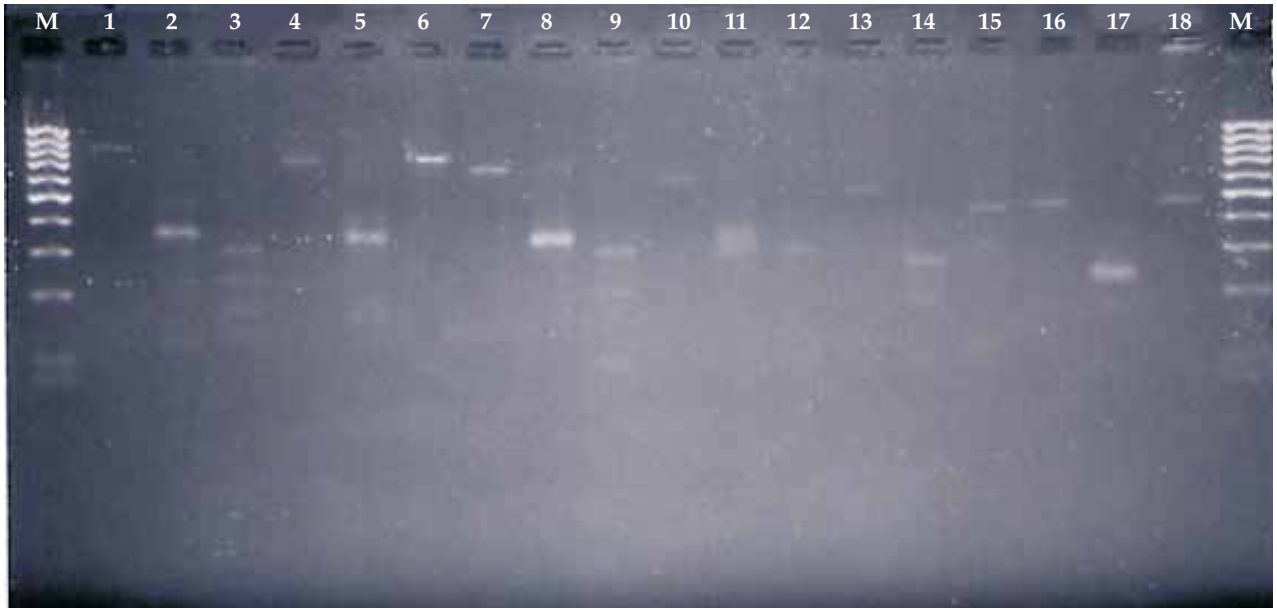

Fig. 3. Agarose gel with PCR products from amplification of the 5.8S-ITS region of rDNA from six different species (Lanes 1,4,7,10,13,16). Digestion products of the amplificates obtained using the restriction enzymes *Hinf*I (Lanes 2,5,8,11,14,17) and *Hae*III (Lanes 3,6,9,12,15,18). Lanes M: Marker 100 bp (1031, 900, 800, 700, 600, 500, 400, 300, 200, 100, 80) (Lanes 1–3: *Saccharomyces cerevisiae*; Lanes 4–6: *H. uvarum*, Lanes 7–9: *K. thermotolerans*, Lanes 10–12: Not identified, Lanes 13–15: *C. vinaria*, Lanes 16–18: *C. stellata*)

spora uvarum was differentiated from *H. guilliermondii* with the use of *Dde*I. On the other hand one strain could not be identified (Lanes 10–12 in Fig. 3).

As described in this paper, PCR amplification of 5.8S-ITS rDNA carefully obtained from fermenting must samples may be a good method for fast identification of the yeasts involved in wine fermentation, yielding information without need of cultivation or time-consuming plate isolation methods.

Conclusion

The method proposed is effective in characterizing the yeast population and detecting potentially undesirable yeasts, such as spoilage yeasts present in early stages of wine fermentation, and can be completed in just a few hours, PCR amplification being the most time-consuming step. Nevertheless further studies are necessary to evaluate the reproducibility of the method.

Acknowledegments

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Identifikacija vinskih kvasaca postupkom PCR-RFLP bez prethodne izolacije na ploči

Sažetak

Populacija vinskih kvasaca tijekom spontane fermentacije mošta karakterizirana je izravnom amplifikacijom regije 5,8S-ITS rDNA bez prethodne izolacije na ploči ili povećavanja količine. Primijenjena je RFLP analiza na svakom utvrđenom amplifikacijskom produktu te je provedena odgovarajuća identifikacija kvasca. Postupak omogućava brz i izravan put utvrđivanja populacije kvasca prisutnog u fermentaciji vina.