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Short communication

Ecology of inoculated and spontaneous fermentations in Rioja (Spain) musts, examined by mitochondrial DNA restriction analysis

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Abstract

Analysis of mitochondrial DNA restriction patterns was used to study the introduction of a selected strain of *Saccharomyces cerevisiae* for fermentation of non-sterile musts of La Rioja (Spain). All of the isolates from the inoculated musts showed the restriction pattern of the selected strain. The same technique was used to study the spontaneous fermentation of musts, showing that a few strains were responsible for the fermentations. One of the strains identified from the spontaneous fermentations had been identified in a previous vintage. © 1997 Elsevier Science B.V.

Keywords: *Saccharomyces cerevisiae*; Mitochondrial DNA restriction analysis; Inoculated fermentations; Spontaneous fermentations

1. Introduction

The use of active dry yeasts to inoculate non-sterile musts is becoming a common practice in most wine-producing regions (Kraus et al., 1983; Reed and Nagodawithana, 1988). The inoculated strain has to compete during fermentation with the natural mixed population of the must. Most of the strains in the must, as well as those used as inocula, belong to the same species, *Saccharomyces cerevisiae*, which makes it difficult to know whether the inoculation has been successful. Therefore, a technique that allows different strains of the same species to be distinguished is necessary.

Mitochondrial DNA restriction analysis was used initially to distinguish between brewing yeasts (Aigle et al., 1984) and has been recently applied to the selection of wine yeasts (Querol et al., 1992b) and to the monitoring of inoculated (Querol et al., 1992a) and spontaneous fermentations (Dubourdieu et al., 1987; Ramón et al., 1990; Frezier and Dubourdieu, 1992; Versavaud et al., 1993).

In this paper, we report on the use of mitochondrial DNA restriction analysis to monitor spontaneous fermentations of Rioja musts, and on a

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study of the introduction of a selected strain in an experimental winery.

2. Materials and methods

2.1. Wine fermentations

Wines were produced from "Viura" grapes harvested during the 1993 vintage in La Grajera (La Rioja, Spain) and processed in the experimental winery of the Centro de Investigaciones Agrarias. The must was supplemented with sulphur dioxide (100 mg l^{-1}), cleared by settling and separated in six stainless steel vessels (250 l each). Three of them were inoculated with the selected strain *S. cerevisiae* V.22, to give a final concentration $1 \cdot 10^5$ cells ml^{-1}. The three remaining tanks were controls. Fermentations were conducted at 17°C for 13–19 days.

2.2. Microbiological analysis

Samples were aseptically taken from each vessel at the following seven stages of fermentation: (1) must, density 1.085; (2) lag phase, density 1.085; (3) exponential growth phase, density 1.080; (4) and (5) vigorous fermentation, densities of 1.070 and 1.010, respectively; (6) end of fermentation, density 1.000 and (7) wine, density 0.991. Appropriate dilutions in sterile peptone water were spread onto plates of glucose–chloramphenicol agar (5 g l^{-1} yeast extract, 20 g l^{-1} glucose, 0.1 g l^{-1} chloramphenicol, 15 g l^{-1} agar). Plates were incubated at 28°C for 48 h. Ten representative yeast colonies were examined from each sample and characterised by mitochondrial DNA restriction analysis. In total, 420 isolates were analyzed as described below.

2.3. Mitochondrial DNA restriction analysis

Mitochondrial DNA analysis was carried out according to the method proposed by Querol and Barrio (1990). Yeasts cells were grown in an overnight culture of 5 ml of YEPD (1% yeast extract, 2% peptone, 2% glucose). Cells were centrifuged by low speed centrifugation (7000 *g* for 5 min), washed twice in 1 ml of distilled water and transferred to a 1.5 microcentrifuge tube. After centrifugation (7000 *g*, 5 min), the pellets were resuspended in 0.5 ml of 1 M sorbitol with 0.1 M EDTA, pH 7.5, and 0.02 ml of a solution of zymoliase 60 (2.5 mg/ml) were added. Tubes were incubated at 37°C for 60 min. Spheroplasts were centrifuged at 7000 *g* for 5 min in a microcentrifuge and resuspended in 0.5 ml of a solution containing 50 mM Tris–HCl, 20 mM EDTA, pH 7.4. After suspension, 0.05 ml of a sodium dodecyl sulfate solution (10% w/v, pH 7.2) was added to each tube and the tubes were incubated at 65°C for 30 min. A 0.2-ml volume of 5 M potassium acetate was added and the mixture was incubated in an ice bath for 45 min and centrifuged at 13 000 *g* for 10 min. Supernatants were transferred to clean microcentrifuge tubes and DNA was precipitated by adding 0.7 ml of isopropanol. After incubation at room temperature for 10 min, the tubes were centrifuged at 13 000 *g* for 10 min. The DNA was washed with 70% ethanol, vacuum dried, and dissolved in 0.05 ml of TE (10 mM Tris–HCl, 1 mM EDTA, pH 7.5). A 2-μl volume of DNA was digested with the restriction endonuclease Alu I, according to the manufacturer's instructions (Boehringer Mannheim). This enzyme recognizes a large number of sites in the yeast nuclear DNA, but only a few sites in mitochondrial DNA. Restriction fragments were separated by electrophoresis in 1% agarose gels and visualized in a UV transilluminator after ethidium bromide staining for 5 min.

All reagents were purchased from Boehringer Mannheim (supplied by Spanish office of Boehringer Mannheim S.A. Biochemica, produced by Boehringer Mannheim, Mannheim, Germany) and Sigma (St. Louis, MO, USA). The electrophoresis system used was a Bio-Rad DNA Sub Cell, (supplied by the Spanish office of Bio-Rad Laboratories and produced by Group Head Quarters, USA) and the power supply was an LKB Biochrom (LKB, Sweden).

3. Results and discussion

In the inoculated vessels, all of the isolates showed a unique restriction pattern (results not shown), coincident with the pattern of the inoculated strain, V.22 (Fig. 1), despite the fact that the must had not been sterilised before inoculation. It is probable

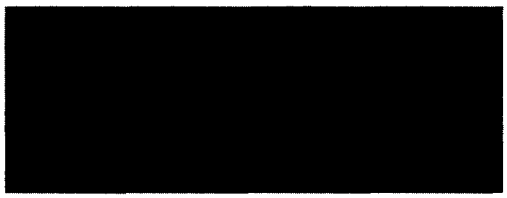

Fig. 1. Mitochondrial DNA restriction pattern of *S. cerevisiae* V.22 treated with the restriction enzyme Alu I. DNA of bacteriophage lambda was treated with the enzymes Eco RI and Hinf III.

that the intense sulphite treatment, which is common practice in the region for white wines, reduced the natural population to such a degree that the inoculated strain was the only actively growing microorganism. Growth and fermentation in non-inoculated tanks were clearly delayed in comparison with the inoculated tanks (data not shown).

Similar results were obtained by Fleurent et al. (1993). They performed six different vinifications inoculated with a selected strain and the prevalence of the strain inoculated was 100% in four of the six fermentations, and 90% in the remaining two. Aziac et al. (1991), on the contrary, observed highly variable prevalences, from 8 to 77%, depending on the winery. These variations were probably due to differences in the technological practices that provoked differences in the ecosystem of the must.

In the spontaneous fermentations, fourteen different strains were detected by mitochondrial DNA restriction analysis (Table 1). In the first steps of the fermentation, seven patterns were present: I, II, III, IV, V, VI and VII (Table 1). Five of these strains, i.e. those corresponding to patterns I, II, III, VI and VII declined towards the final steps of the fermentation in favour of patterns IV (which was only sporadically isolated in the initial steps) and X (which was not detected until the end of the fermentation). Only pattern V was present throughout the entire fermentation process in most of the tanks, although it was present at low levels in the early stages, however, its contribution increased significantly during the fermentation process. In fact, it was the most abundant strain at the end of fermentation, comprising more than 50% of all isolates during vigorous fermentation and in the finished wine. Patterns VII, VIII, IX, XI, XII and XIII were rarely isolated. Finally, the remaining pattern, XIV, was present in the uninoculated must only.

We can conclude that a few strains were responsible for the fermentation of the non-inoculated

Table 1
Percentage of the different mitochondrial DNA restriction patterns during spontaneous fermentations of D.O.C. Rioja musts from La Grajera (La Rioja, Spain)

Patterns	Density																		
	Must	1.085			1.080			1.070			1.010			1.000			0.991		
		A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
I	10	40	30	30	30	40	50	40	40	10	10	0	10	0	0	0	0	10	0
II	10	10	0	20	10	20	0	0	0	0	0	10	0	0	0	0	0	0	0
III	0	0	0	10	30	20	0	10	20	10	0	10	0	0	0	0	0	0	0
IV	0	10	0	10	0	0	0	10	10	30	20	0	0	20	40	40	20	20	0
V	0	10	0	0	20	0	20	30	10	50	30	70	70	40	40	60	30	40	90
VI	0	20	70	30	10	20	0	0	10	0	40	0	0	0	0	0	0	0	10
VII	0	10	0	0	0	0	20	0	0	0	0	0	0	0	0	0	0	0	0
VIII	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0
IX	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	10	0	0
X	0	0	0	0	0	0	0	0	0	0	0	10	0	0	20	0	40	20	0
XI	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0
XII	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0
XIII	0	0	0	0	0	0	0	0	0	0	0	0	10	40	0	0	0	0	0
XIV	80	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

A, B and C: Repetitions in three independent tanks.

musts, as has been observed in similar studies by Querol (1992), in Alicante (Spain), as well as by Fleurent et al. (1993) and Versavaud et al. (1993) in Charentes (France).

It is interesting to note that pattern III, corresponding to strains isolated from the spontaneous fermentations, was similar to the restriction pattern of the selected strain, V.22 (Fig. 2). The latter was originally isolated in the experimental winery of the Centro de Investigaciones Agrarias of La Rioja in 1990 and, although it was not dominant during the spontaneous fermentations studied here, its appearance could be interpreted as being a stable typical strain of the winery, according to investigations by Frezier and Dubourdieu (1992) and Vezinhet et al. (1992). Despagne (1991) has called attention to the risk of contamination from machinery used in the winery, however, in our study, special care was taken to avoid contamination. *Saccharomyces cerevisiae* V.22 was isolated originally in 1990 from must from "La Grajera", the same vineyard from which we obtained the grapes for this study (in 1993).

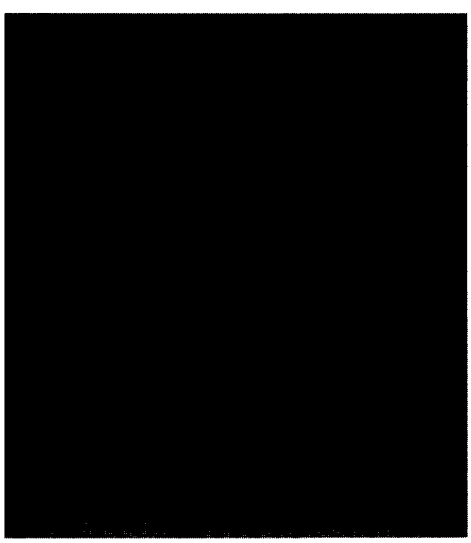

Fig. 2. Mitochondrial DNA restriction patterns of the most abundant strains in spontaneous fermentations of musts from La Grajera (La Rioja, Spain), treated with the restriction enzyme Alu I. DNA of bacteriophage lambda was treated with the enzymes Eco RI and Hinf III (F).

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