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Ecology of spontaneous fermentation in one winery during 5 consecutive years

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A.R. GUTIÉRREZ, P. SANTAMARÍA, S. EPIFANIO, P. GARIJO AND R. LOPEZ. 1999. The ecology of spontaneous fermentation in a new winery in La Rioja (Spain) was studied during 5 consecutive years using mitochondrial DNA restriction analysis. The number of different strains detected for each vintage and their appearance frequency varied from one year to another. A small number of strains were present in consecutive years, but the presence of each one varied in function of the specific year. Only one strain was present in all the 5 years studied. For the 1997 vintage, an unusual dominance of non-*Sacharomyces* yeasts in the vigorous fermentation was detected; this may explain the abnormal analytical data for the wines of that year.

INTRODUCTION

Spontaneous alcoholic fermentation of grape must is a complex process carried out by the sequential action of different yeast genera and species. The composition of the yeast flora can vary according to the climatic conditions (Parrish and Carroll 1985), the grape variety (Schütz and Gafner 1994) and the vinification technology (Charoenchai *et al*. 1998). Indigenous yeasts with low fermentative power of the *Kloeckera*, *Hanseniaspora*, *Candida* and *Pichia* genera grow during the early stages of many fermentations; when the ethanol concentration increases, the more ethanol-tolerant *Saccharomyces* species complete the fermentation (Fleet and Heard 1993).

Preliminary investigations have shown the presence of mixed populations of different strains of *Saccharomyces cerevisiae* involved in spontaneous fermentations (Nadal *et al*. 1996). Several genetic methods for yeast strain identification have demonstrated that there is a great genetic diversity in enological strains of *S. cerevisiae*, e.g. analysis of mitochondrial DNA (Constantí *et al*. 1997), pulsed field gel electrophoresis (Izquierdo *et al*. 1997), DNA fingerprinting by RAPD–PCR (Molnar *et al*. 1995). This diversity is affected by geographical location (Guillamón *et al*. 1996) and by the technology used in vinification (Epifanio *et al*. 1999).

The equilibrium of different micro-organisms present in the initial flora, the succession order between species and the diversity in *Saccharomyces* strains can all vary between different years (Querol 1992), so explaining the differences in fermentation rate and wine characteristics.

In recent years, much work has been carried out on the ecology of wine yeasts in order to study whether spontaneous fermentations are conducted by a high or low number of strains of *S. cerevisiae* and whether there is stability of yeast strains in the wineries from one year to another (Frezier and Dubordieu 1992; Vezinhet *et al*. 1992); differing results have been obtained. These studies are of great interest in order to establish the existence of typical strains belonging to one ecosystem and also to carry out selection programmes of these representative yeasts; these would then be useful as inocula in the vinifications carried out in that specific enological area.

The aim of this work was to study the ecology of spontaneous fermentations in one winery during 5 consecutive years and also to study the differences between the years on the microflora composition and kinetics of alcoholic fermentation.

MATERIALS AND METHODS

Vinifications

Spontaneous fermentations were carried out during the 1994, 1995, 1996, 1997 and 1998 vintages in the same cellar (a new winery established in 1994), using free-running white grape juice of the Viura variety from La Rioja, Spain. Sulphur dioxide (50–60 mg l^{-1}) was added to the musts before the start of fermentation. The fermentation temperature was con-

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trolled at 18 °C. Dry yeast was not used. The fermentations were carried out in 100-l stainless steel tanks. The number of tanks included in the study was 12 in 1994, 10 in 1995, six in 1996 and seven in 1997 and 1998.

Isolation and identification of yeasts

Samples were taken from all tanks during two stages of fermentation: vigorous and final. The samples were plated out onto chloramphenicol glucose agar (Biokar Diagnostics, Beauvais, France) at a serial decimal dilution and incubated at 28 °C for 2 days. Plates containing between 30 and 300 colonies were examined; 10 colonies from each sample were selected and analysed randomly. This meant that only those strains present in every tank at a percentage higher than, or equal to, 5% were detected. The data from all the tanks in each year were treated together. There were 240 isolates in 1994, 200 in 1995, 120 in 1996 and 140 in 1997 and 1998. Every year 10 colonies were isolated from the musts before their division into lots and fermentation; these were also studied.

Mitochondrial restriction analysis (Guillamón 1996) were used to determine that the isolates were *Saccharomyces*. Individual strains were identified by mitochondrial DNA (mtDNA) restriction analysis (Querol *et al*. 1992). The different patterns obtained were designated with Roman numerals. Subscripts a, b, c, d, e correspond to 1994, 1995, 1996, 1997 and 1998, respectively.

Wine analysis

The wines were analysed according to official EU methods (Anonymous 1990).

RESULTS AND DISCUSSION

The mtDNA restriction patterns of the isolates taken from the musts showed the absence of *Saccharomyces* strains before the fermentations began. At this stage, we studied 50 strains (10 per year) and none of them belonged to this genera. Other studies (Rosini *et al*. 1982) had indicated previously that the *Saccharomyces* genera is rarely present at the start of fermentation. However, all the colonies from the fermentative stages were *Saccharomyces*, apart from some isolates proceeding from the 1997 vintage. In this year, as we will see below, the fermentations were extremely problematic. The data clearly show the rapid adaptation and domination in the fermentative medium of the *Saccharomyces* strains.

The mtDNA restriction patterns of the 840 isolates from the fermentative stages for the 5 years revealed 72 different profiles; 70 of them corresponded to different *S. cerevisiae* strains.

The number of different strains detected for each vintage and their appearance frequency varied from one year to another (Table 1). There was no one clearly dominant clone in the fermentation for those years with a high number of different patterns (1995 and 1997). For the other 3 years, a clear majority strain was detected. In 1994, pattern VIII**a** was significantly present at a frequency of 30%; in 1996, pattern II_c and in 1998, pattern I_e were the dominant strains with percentages of 56% and 59%, respectively. Every year there were a high number of patterns whose presence was smaller than 5%.

These two different results, a large number of strains at low percentages or a smaller number of strains with one dominant, has been reported previously. Querol *et al*. (1994) compared the dynamics in the yeast populations of two different wineries and observed that in one of them there was a dominant strain and in the other there was a succession of clones present to a very small extent. Versavaud *et al*. (1995) detected, in all the vinifications studied, that fermentations were managed by one or two strains and that these were more than 50% of the total population. Both situations were found in this study, depending on the specific year studied. It is not possible, therefore, to say that there is only one valid possibility for all cases; the situation can differ, as in the above studies, in function of the winery or enological zone or, as in our case, in function of the specific year.

When the mtDNA restriction patterns of the strains in the five vintages were compared, it was observed that some of them were present in consecutive years (Table 2). Pattern $VIII_a$ in 1994 is identical to patterns I_b, $VIII_c$, IV_d and V_e from 1995, 1996, 1997 and 1998, respectively. Something similar happened with pattern XIV_a from 1994, which appeared in all the years with the exception of 1997, an unusual year. The amounts of these common patterns showed a great variability in function of the specific year studied. Pattern $VIII_a$ from 1994 had an appearance frequency of 30%; in the other 4 years it appeared at levels lower than 10%. Something similar happened with pattern II_c from 1996 and pattern I_e from 1998. These two strains were dominant in those years; in the other years when they were present, they showed a very low level. The remaining patterns common to several vintages appeared to a very small extent.

Vezinhet *et al*. (1992), reported on the existence of representative strains of one ecosytem or the 'winery effect'; that is, the retention of strains in the winery from one year to another. The data from this work showed the existence of 13 strains which appeared in more than 1 year; only one of them was present in all of the 5 years studied. The present study also showed that the significance of these common strains in vinification varies from one year to another according to the elaboration conditions, principally the composition of the

Table 1 *Saccharomyces* patterns and total frequencies of each fermentation studied. The data is the average of the vigorous and final stages of fermentation in 12 tanks in 1994, 10 in 1995, six in 1996 and seven in 1997 and 1998

1994		1995		1996		1997		1998	
Pattern	%	Pattern	%	Pattern	%	Pattern	%	Pattern	%
I_a	11	I_b	10	I_c	1	I_d	4	I_e	59
II_a	7	II_b	3	II_c	56	II_d	13	II_e	4
III_a	16	III_b	1	III_c	1	III_d	15	III_e	9
IV_a	2	IV_b	1	IV_c	1	IV_d	6	IV_e	4
V_a	2	V_b	13	V_c	1	V^*_d	21	V_e	6
VI_a	8	VI_b	4	VI_c	13	VI^*_d	4	VI_e	4
VII_a	4	VII_b	13	VII_c	6	VII_d	8	VII_e	1
$VIII_a$	30	$VIII_b$	1	$VIII_c$	3	$VIII_d$	2	$VIII_e$	1
IX_a	1	IX_b	2	IX_c	2	IX_d	3	IX_e	6
X_a	2‘	X_b	3	X_c	4	X_d	1	X_e	1
XI_a	2	XI_b	5	XI_c	1	XI_d	4	XI_e	1
XII_a	1	XII_b	2	XII_c	2	XII_d	6	XII_e	1
$XIII_a$	2	$XIII_b$	3	$XIII_c$	1	$XIII_d$	3	$XIII_e$	1
XIV_a	6	XIV_b	1	XIV_c	1	XIV_d	1	XV_a	2
Xv_a	2	XV_b	1	XV_e	1	XV_d	1	XV_c	1
XVI_a	1	XVI_b	6	XVI_c	1	XVI_d	1		
$XVII_a$	3	$XVII_b$	10	$XVII_c$	1	$XVIII_d$	1		
		$XVIII_b$	6	$XVIII_c$	1	$VIII_d$	1		
		XIX_b	6	XIX_c	3	XIX_d	1		
		XX_b	1			XX_d	2		
		XXI_b	4			$XVII_d$	1		
			$XXII_b$		4			$XXII_d$	1

*Non-*Saccharomyces* strains.

Table 2 Identical clones isolated in different vintages and the appearance frequency of each (patterns on the same line are identical)

1994		1995		1996		1997		1998	
Pattern	%	Pattern	%	Pattern	%	Pattern	%	Pattern	%
II_a	7					$VIII_d$	2		
III_a	16	$XVII_b$	10			VII_d	8		
IV_a	2					X_d	1		
V_a	2	XI_b	5						
VI_a	8							II_e	4
$VIII_a$	30	I_b	10	$VIII_c$	3	IV_d	6	V_e	6
XIV_a	6	II_b	3	V_c	1			$XIII_e$	1
XV_a	2			II_c	56			VI_e	4
XVI_a	1	$XIII_b$	3					I_e	59
		XVI_b	6	VII_c	6	I_d	4		
		XIX_b	6	IX_c	2	IX_d	3		
				$XIII_c$	1			XV_e	1
				$XVIII_c$	1	XII_d	6		

grape must (variable every year). Therefore, for our winery, it is not possible to affirm that typical strains of *S. cerevisiae* exist that play an important role in all the vinifications. Schütz and Gafner (1994) studied the ecology in one winery over two consecutive vintages and they indicated that the population of yeasts differed widely from one year to another. These results would be more in accordance with those obtained in the present work. It should be emphasized that this winery started to produce wine in 1994, and that the creation of an ecosystem made up of specific strains may require a longer period. We therefore think that ecological studies should be carried out over several more consecutive years in order to further clarify the developments taking place in this winery.

Some authors have suggested that a selection programme could be based exclusively on those strains which appear as dominant in some vinifications, or which are present in successive vintages within one ecosystem (Sabate *et al.* 1998). From the above results, we believe that this criteria is not sufficient. This study shows that very few strains are common to several years (from a very wide clonal diversity) and that strains which appear as dominant in one year may completely disappear in another. The above-mentioned selection programme criteria would suppose the loss of a very large genetic potential and the elimination in advance of strains which could be very useful in determined vinification conditions (Epifanio *et al.* 1999).

In the 5 years studied, 4 had normal fermentations; the exception was the 1997 vintage. This year was extremely problematic in many Spanish areas of wine production, because many stuck fermentations were detected. The wines elaborated in 1997 did not finish the fermentations and their volatile acidity was excessively high (average data of residual sugars and volatile acidity were 16·78 g/l and 2·56 g/l, respectively). As described previously (Dubois *et al.* 1996), fermentation kinetics change significantly with year and maturity.

This unusual situation is also reflected in the microbiological data obtained in 1997. Non-*Saccharomyces* yeasts were present as dominant strains in the samples studied during the vigorous fermentation stage (Table 3). Patterns V and VI (non-*Saccharomyces*) were present in vigorous fermentation at frequencies of 42% and 8%, respectively. Both patterns disappeared in the final stage of fermentation and they were replaced by a high number of clones belonging to the *Saccharomyces* genera. These new strains were not present in the vigorous stage and, at the end of the fermentation, each one was present to a very small extent. Figure 1 shows the restriction profiles of some clones isolated in 1997; a typical non-*Saccharomyces* profile for strain V can be seen (Guillamón 1996). The imbalance in the normal succession in the yeast population, with the presence of non-*Saccharomyces* strains as the predominant yeasts in 1997, may explain the sticking and the abnormal analytical data for the wines.

Table 3 Evolution of different strains (%) during 1997 vinifications

Strains	Vigorous fermentation	Final fermentation	Total
I	4	4	4
II	9	17	13
III	18	12	15
IV	6	6	6
V*	42	–	21
VI*	8	–	4
VII	4	12	8
VIII	3	1	2
IX	2	4	3
X	2	–	1
XI	–	8	4
XII	1	11	6
XIII	–	6	3
XIV	–	2	1
XV	–	2	1
XVI	–	2	1
XVII	–	2	1
XVIII	–	2	1
XIX	–	2	1
XX	1	3	2
XXI	–	2	1
XXII	–	2	1

*Non-*Saccharomyces* strains.

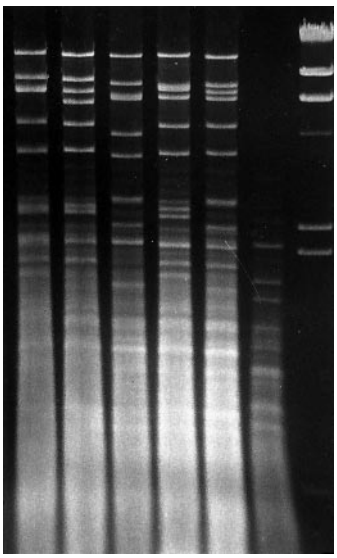

Fig. 1 Mitochondrial DNA electrophoresis of some yeasts isolated in the 1997 vintage using the restriction enzyme *Alu*I. Lanes 1–5 correspond to different *S. cerevisiae* strains. Lane 6 corresponds to pattern V and shows the typical non-*Saccharomyces* profile. Lane 7 corresponds to phago lamda cut with *Hind*III.

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