Growth of Natural Flora during the Fermentation of Inoculated Musts from "Pedro Ximenez" Grapes

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The growth of naturally occurring micro-organisms in musts from grapes collected at three degrees of ripeness (unripe, half-ripe and ripe) in the Montilla-Moriles (Southern Spain) region during 1985 and 1986 was studied. The musts were inoculated with four physiological races of *Saccharomyces cerevisiae* isolated in the region. Although the inoculated races of *S. cerevisiae* prevailed throughout the fermentations, there was also a significant growth of indigenous races of *S. cerevisiae* and a less marked growth of other micro-organisms such as bacteria, filamentous fungi and yeasts other than *S. cerevisiae*.

Wine makers have traditionally relied on the fermentation of grape musts by yeasts originally present in the grapes or in the cellar. Yeasts of the genera *Kloeckera, Candida, Pichia, Hansenula, Rhodotorula* and *Torulaspora* grow during the first few days of fermentation and disappear during the later stages, when prevailing *Saccharomyces cerevisiae* strains, complete the process (Iñigo and Arroyo, 1960; Benda, 1962; Khayyat *et al.*, 1982; Mauricio *et al.*, 1986; Fleet *et al.*, 1984). As a whole, the non *S. cerevisiae* species produce a broader spectrum of aroma compounds (Amerine *et al.*, 1982; Benda, 1981), which is desirable in regions with well-established enological features.

The inoculation of musts with selected indigenous yeasts can offer advantages such as faster and more controlled fermentations and the production of wines of more uniform quality provided they prevail over the naturally occurring flora, whose growth is then kept within moderate bounds. Heard and Fleet (1985) studied the growth of natural flora in inoculated grape musts but failed to determine whether the *S. cerevisiae* strain prevailing at the end of fermentation was that originally inoculated or if, on the contrary, it was a naturally occurring strain which took over the process.

In this work we studied the growth of natural flora in wines inoculated with four indigenous races of *S. cerevisiae* isolated in the Montilla-Moriles region by identification of their colonies in Agar-Bismuth Sulphite medium in order to check whether the inoculated races prevailed over the indigenous yeasts of the musts throughout the fermentation.

MATERIALS AND METHODS

The experiments were carried out on musts obtained from grapes of the *Vitis vinifera* "Pedro Ximénez" variety collected at three degrees of ripeness, namely unripe (must I), half-ripe (must II) and ripe (must III), in the 1985 and 1986 vintages. The diminishing sugar concentration of the musts was determined by density measurements and is listed in Table 1.

Although the musts were not sterilized, half their volume was centrifuged in order to reduce their suspended solid fraction to about 3,5% (W/V). Sulphur dioxide at a concentration of 120 mg/*l* and enough tartaric acid to adjust the pH to 3,2 were added; these conditions are thought to be those prevailing in the fermentations carried out in the region (Mauricio *et al.*, 1986).

The yeasts used in the inocula were four physiological races of *Saccharomyces cerevisiae, viz.* strains *cerevisiae* A (maltose: ferm, acid and gas), *cerevisiae* B (maltose: ferm. acid Assim. +), *chevalieri* and *capensis* (Kreger van Rij, 1984).

The musts were inoculated with a 2% (V/V) inoculum of pure cultures (36-h old) of each of the selected yeasts. Races *cerevisiae* A and B were used to ferment the musts of the 1985

TABLE 1
Sugar concentration in the musts and alcoholic degree reached in each fermentation

Year	Must	Sugar content (g/*l*)	*S. cerevisiae* races used			
			cerevisiae A	*cerevisiae B*	*chevalieri*	*capensis*
			Ethanol content (% V/V) in the wines			
1985	I	170,85	9,85	9,31		
	II	226,90	11,58	12,83		
	III	276,00	14,34	14,00		
1986	I	178,00			10,07	10,39
	II	215,88			12,59	12,5
	III	231,89			12,69	13,15

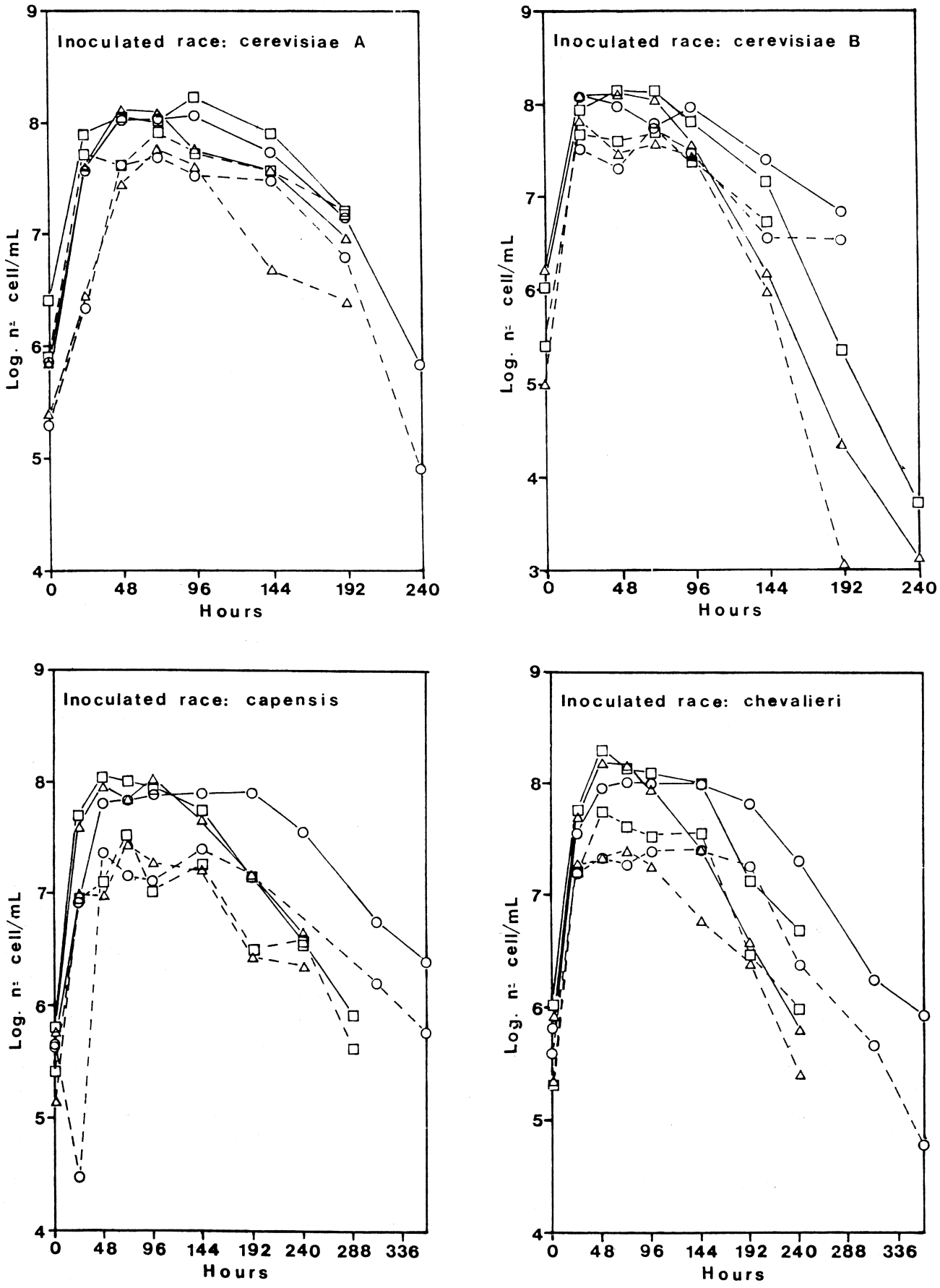

FIGURE 1

Growth of inoculated race of *S. cerevisiae* (fine lines) and total indigenous yeasts (dashed lines) during the fermentation of the three types of musts: I (△)II (□) and III (○).

vintage, while races *chevalieri* and *capensis* acted on the musts of the 1986 vintage.

All fermentations were carried out at 25°C, the most suitable temperature for the fermentations carried out in the region (Moreno *et al.*, 1986). Samples were periodically taken until fermentations were completed (Table 2).

The total number of viable micro-organisms (yeasts, bacteria and filamentous fungi) was determined by seeding into YM Agar (Wickerham, 1951). The number of *S. cerevisiae* of each inoculated race was determined by seeding in Biggy Agar (Difco). This medium contains bismuth sulphite, which yields a brown precipitate with hydrogen sulphide which allows one to distinguish between colonies according to their colouration: the more H_2S they produce and store, the darker they appear (Rupela and Tauro, 1984). In our experiments, the darkest colouration was yielded by the strain *cerevisiae* B, followed by *chevalieri* - which, in addition, grew more slowly in this medium-, *cerevisiae A, and capensis.*

The number of indigenous yeasts other than *S. cerevisiae* was determined by seeding in Yeast Carbon Base (Difco), containing lysine as sole nitrogen source. None of the four *S. cerevisiae* races selected grew in this medium. The indigenous yeasts which were isolated were identified according to Kreger van Rij (1984).

The alcoholic contents of the fermented grape musts were determined by the method of Crowell and Ough (1979).

RESULTS AND DISCUSSION

Figure 1 shows the evolution of the growth of the four *S. cerevisiae* races inoculated and that of total indigenous yeasts throughout the fermentations. The number of yeasts from the inoculum was substantially greater than that of indigenous yeasts throughout the fermentation. The indigenous yeasts grew appreciably during the first few days and therefore probably contribute to some extent to the bouquet of the end product. The maximum population of the inoculated yeast as well as the total indigenous yeast was reached within the first 48-72 hours. As a rule, the maximum concentration was kept for a longer time in must III - with the highest sugar concentration - than in must I (unripe grapes), which testifies to a lengthier fermentation in the former. As far as the inoculated races are concerned, *capensis* remained suspended for the longest time, followed by *chevalieri, cerevisiae* A and *cerevisiae* B - this was also the order of fermentation rate, from lowest to highest (Millán and Ortega, 1988).

The indigenous yeast population consisted mainly of *S. cerevisiae* races, unable to grow in lysine-agar, together with species other than *S. cerevisiae* which do grow in this medium. The latter accounted for a small fraction which, except at the beginning of the fermentation, never reached levels of 10^4 cells/m*l* (Table 6).

Tables 3-5 list the percent frequencies of the samples where bacteria, filamentous fungi and yeasts of different species were detected on the lysine-agar medium. Table 6 gives the average numbers of these micro-organisms in all positive samples.

Bacteria were isolated during the fermentations until the ethanol concentration reached 10-12% (V/V), above which none was detected (Tables 3-5). Their concentration decreased as the alcohol content increased (Table 6). Filamentous fungi were detected throughout the fermentation (Tables 3-5); their concentrations were particularly high for ethanol contents between 0 and 2% (V/V), and decreased dramatically above 4% ethanol (V/V) (Table 6). These results are consistent with those reported by García Maiquez (1978) for Jerez wines.

Candida stellata was the indigenous yeast most frequently isolated in all the fermentations (Tables 3-5). Its concentration decreased particularly at ethanol contents above 6-8% (V/V) (Table 6). This osmophilic yeast is frequently isolated from grapes (Sapis-Domercq and Guittard, 1976; Benda, 1981) and occurs even more frequently in grapes infected by *Botrytis cinerea* (Minarik *et al.*, 1978; Le Roux *et al.*, 1973).

Kloeckera apiculata was not detected in the musts from unripe grapes, though it was indeed isolated with a high frequency in those from half-ripe and ripe grapes, particularly in the early stages of fermentation (Tables 3-5), which appears to indicate that this yeast, reported as one of the most common at the beginning of spontaneous grape must fermentations (Benda, 1981), only multiplies in grapes in their final stage of ripening. Its concentration in the wine diminished dramatically at ethanol contents above 10% (V/V) (Table 6).

The two *Rhodotorula* species isolated, namely *Rh. rubra* and *Rh. glutinis,* were detected only sporadically, with the exception of the latter in the musts from ripe grapes of the 1986 vintage, where it was found to occur with higher frequencies.

Zygosacharomyces bailii was only isolated in the late stages of fermentation, particularly in the wines with the highest alcohol contents (must III). This shows their high tolerance to ethanol, which, according to Van der Walt (1970), is a result of the singular properties of its cytoplasmic membrane. The occurrence of this yeast in grape musts has been reported by various authors. Benda (1981) regards it as an inducer of veil and turbidity, though it has never been shown to alter wines.

Candida guilliermondii was only isolated at the beginning of fermentation, while *Pichia membranaefaciens* was detected almost exclusively in the musts from half-ripe grapes of the 1986 vintage. Its concentration fluctuated up to an ethanol content of 8% (V/V), above which it started to decrease sharply. Both yeasts are aerobic and liable to produce detrimental veils on the surface of wines.

Our results show that yeasts naturally occurring in musts - particularly indigenous races of *S. cerevisiae* - contribute significantly to the fermentation of inoculated wines and, according to Benda (1981) and Fleet *et al.* (1984), exert a major influence on wine bouquet. However, the inoculated yeasts prevailed over their indigenous counterparts in all the fermentations; they resulted in fast fermentations (Martínez *et al.*, 1988) and partly inhibited the growth of the latter, especially that of species other than *S. cerevisiae* - and particularly those considered to be detrimental.

Thus, the inoculation of musts with strains of *S. cerevisiae* selected among indigenous yeasts occurring naturally in the winemaking area can contribute to a better control of the alcoholic fermentation of wines and allow only partial growth of the remaining indigenous yeasts.

TABLE 2
Distribution of samples during the fermentation of the three types of must

Ethanol content (% V/V)	Number of samples taken			
	Must I	Must II	Must III	Total
0-2	7	6	7	20
2-4	2	3	4	9
4-6	3	2	3	8
6-8	4	5	2	11
8-10	13	1	2	16
10-12	2	9	4	15
12-14		6	8	14
14-16			5	5
Total	31	32	35	98

TABLE 3
Frequency of isolation (%) in the samples grown in Lysine-Agar during the fermentations carried out on musts from unripe grapes

Species	Ethanol content (% V/V)					
	0-2	2-4	4-6	6-8	8-10	10-12
Candida stellata	86	100	100	75	23	0
Kloeckera apiculata	0	0	0	0	0	0
Rhodotorula rubra	0	0	0	0	8	0
Rhodotorula glutinis	0	0	33	0	8	0
Zygosaccharomyces bailii	0	0	0	0	15	0
Candida guilliermondii	29	0	0	0	0	0
Pichia membranaefaciens	0	0	0	0	0	0
Filamentous fungi	71	0	66	25	23	100
Bacteria	28	100	66	75	15	0
Unidentified yeasts	29	50	100	75	31	0

TABLE 4
Frequency of isolation (%) in the samples grown in Agar-Lysine during the fermentations carried out on musts from half-ripe grapes

Species	Ethanol content (%V/V)						
	0-2	2-4	4-6	6-8	8-10	10-12	12-14
C. stellata	100	100	100	100	100	100	17
K. apiculata	50	66	100	60	0	22	0
Rh. rubra	0	0	0	0	0	22	33
Rh. glutinis	0	0	0	0	0	11	0
Zygosaccharomyces bailii	0	0	0	0	0	0	17
C. guilliermondii	0	0	0	0	0	0	0
P. membranaefaciens	17	33	100	60	0	22	0
Filamentous fungi	83	100	50	40	100	44	66
Bacteria	0	33	50	20	0	0	0
Unidentified yeasts	50	100	100	80	100	78	17

TABLE 5
Frequency of islolation (%) in the samples grown in Agar-Lysine during the fermentations carried out on musts from ripe grapes

	Ethanol content (%V/V)							
Species	0-2	2-4	4-6	6-8	8-10	10-12	12-14	14-16
C. stellata	57	100	100	50	50	50	25	0
K. apiculata	14	100	100	0	100	50	12	0
Rh. rubra	0	0	0	0	0	0	0	0
Rh. glutinis	43	100	33	0	50	0	0	0
Zygosaccharomyces bailii	0	0	0	0	50	50	75	0
C. guilliermondii	29	0	0	0	0	0	0	0
P. membranaefaciens	0	25	0	0	0	0	0	0
Filamentous fungi	85	0	0	100	0	25	12,5	60
Bacteria	14	100	100	50	100	50	0	0
Unidentified yeasts	14	0	0	50	0	25	0	0

TABLE 6
Average concentration (c.f.u./m*l*) of bacteria, filamentous fungi and yeasts other than *S. cerevisiae* in the samples with positive isolation in Lysine-Agar in the three types of must

	Ethanol content (% V/V)							
Species	0-2	2-4	4-6	6-8	8-10	10-12	12-14	14-16
C. stellata	1470	464,34	434,45	467,68	105,24	222,19	55,53	0
K. apiculata	1706	609,42	578,66	497,53	233,30	42,48	10,0	0
R. rubra	0	0	0	0	20	6	6	0
Rh. glutinis	2155	2991,63	16,65	0	8,30	3,30	0	0
Zygosaccharomyces bailii	0	0	0	0	266,65	266,65	876,6	0
C. guilliermondii	5863	0	0	0	0	0	0	0
P. membranaefaciens	800	176,60	329,95	212,2	0	74,95	0	0
Filamentous fungi	1334,43	202,10	6,65	46,07	12,83	6,51	7,72	12,10
Bacteria	1144,20	746,64	259,60	99,24	47,50	28,3	0	0
Unidentified yeasts	937,7	114,7	204,30	645,65	114,84	30,25	13,3	0

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