Enological Behaviour of Biofilms Formed by Genetically-Characterized Strains of Sherry *Flor* Yeast

María E. Rodríguez[1,†], Juan J. Infante[2,†], Juan J. Mesa[3,†], Laureana Rebordinos[1], and Jesús M. Cantoral[1,*]

[1]*Laboratorio de Microbiología y Genética, CASEM, Departamento de Biomedicina, Biotecnología y Salud Pública. Universidad de Cádiz. Polígono Río San Pedro s/n, 11510 Puerto Real, Cádiz, Spain*

[2]*Bioorganic Research and Services, S.L.-Bionaturis, Centro Andaluz de Biología del Desarrollo, Sevilla, Spain*

[3]*Bodegas Barbadillo S. L. C/ Luis Eguilar, 11540, Sanlúcar de Barrameda, Cádiz, Spain*

Abstract: The *flor* yeasts (*Saccharomyces cerevisiae*) form a biofilm, known as *flor velum*, on the surface of *fino*-type sherry wine at the end of the alcoholic fermentation. These film-forming yeasts are responsible for the oxidative transformation of alcohol to acetaldehyde, together with other reactions, which produce the characteristic flavours and aromas of these wines. In this study, we examine the enological behaviour of eight *flor* yeast strains biofilms in biological aging experiments carried out in the laboratory. Strains with identical chromosomal and mitochondrial DNA patterns and the same origin showed a more closely-related enological behaviour. But the kinetics of growth and acetaldehyde accumulation in the wine were found to be strain-dependent. Moreover, some strains were marked by high acetaldehyde accumulation in their pure cultures during the various phases of the biofilm development. These results provide valuable knowledge for planning technical strategies to improve the biological aging process in the sherry wine industry.

Keywords: *flor* yeast, sherry wines, pure culture, biofilm growth, acetaldehyde.

INTRODUCTION

The sherry wine-making process is characterized by the addition of alcohol to the freshly-fermented wine to raise the concentration of ethanol to 15% (v/v). The wine, which is depleted in fermentable sugars after the fermentation phase, is then aged in partially filled 600 L oak barrels. During the aging process a spontaneous biofilm of yeast (*flor* yeast) grows on the surface of the wine inside the barrels.

The initial studies conducted to differentiate between strains of *flor* yeasts tested the ability of the yeast clones to assimilate and ferment different sugars. Based on these criteria, the *flor* yeasts of the Jerez region of South-western Spain were split into four different races of the *S. cerevisiae* species, denominated *beticus*, *cheresiensis*, *montuliensis*, and *rouxii* [1]. Since then, molecular methods have been used to characterize the *flor* and other industrial yeasts of the Jerez region [2-6]. These techniques are based on polymorphisms in the number and size of chromosomes and on the restriction patterns of the mitochondrial DNA (mtDNA). However, although these molecular techniques have been available to the wine producers for several years, the biological aging of Sherry wines is not yet subjected to microbiological control in the wineries.

The aerobic metabolism of *flor* yeasts influences the characteristics flavour and aroma compounds of the finished sherry [7]. In particular, the acetaldehyde released into the wine by the yeast is considered to be the most important index of aging in sherry wines [8].

The length of the aging period and the large number of oak barrels required in the industrial system represent considerable costs for the producer. One of the more significant costs is that associated with the periodic replenishment of the ethanol consumed by the *flor* yeasts. This action is critical to prevent the wine from being spoilt by other biofilm-forming yeasts that might grow at ethanol concentrations lower than 15% (v/v). The optimum length of the aging period could be shortened by selecting *flor* yeast strains capable of inducing a more rapid accumulation of the metabolites responsible for the sensory properties of the wine, and then using these strains to inoculate all the wine aging in the winery's barrels. Therefore, the potential association of specific molecular patterns with differences in the performance of the *flor* yeasts under industrial conditions suggests that molecular methods might be particularly appropriate for identifying and monitoring *flor* yeast strains with the object of controlling and improving the aging process. This possibility has also been supported by the molecular characterization of different sherry wine aging systems reported by other authors [9, 10].

*Address correspondence to this author at the Laboratorio de Microbiología y Genética, CASEM, Departamento de Biomedicina, Biotecnología y Salud Pública. Universidad de Cádiz. Polígono Río San Pedro s/n, 11510 Puerto Real, Cádiz, Spain; Tel.: +34 956 01 61 56; Fax: +34 956 01 61 80; E-mail: jesusmanuel.cantoral@uca.es

†These authors have contributed equally to this work.

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In this study, we selected eight strains of *Saccharomyces cerevisiae flor* yeast, which had previously been defined on the basis of their electrophoretic karyotypes and mitochondrial DNA restriction profiles, in order to examine the behaviour of the biofilm in pure cultures during a long period of sherry wine aging. We carried out the assays under laboratory conditions imitating the industrial process. The accumulation of acetaldehyde in the wine, the consumption of ethanol and volatile acidity, growth kinetics and development of the biofilm were the critical aspects of the process analyzed. We then examined the correspondence between enological behaviour and molecular patterns of the strains. Based on the results of this study, we propose a strategy for improving the industrial biological aging *fino*-type sherry wines by selecting the most suitable *flor* yeast strain.

MATERIALS AND METHODS

Yeast Strains

The *S. cerevisiae* yeast strains used in this study and their origin are shown in Table **1**. The eight yeasts were isolated from both static and dynamic industrial aging systems in a winery located at the Jerez region in southern Spain. Añada A and Añada B were two static systems for aging the sherry, with a wine of young age; the second and fifth criaderas were part of a dynamic aging system.

Wine Conditions

In this study the wine inoculated with the *flor* yeast strains was obtained from a winery in the Jerez region (Cadiz, Spain). It was a dry (*fino*) sherry wine, previously fermented and fortified, with the following characteristics: $15.52 \pm 0.18\%$ (v/v) ethanol, 81.8 ± 10.3 mg/L acetaldehyde, 0.38 ± 0.02 g/L volatile acidity and pH 3 ± 0.04 (avg $\pm$ SD of the different samples supplied).

Biological Aging of Sherry Wine in Pure Cultures

Yeast cells were grown in 200 mL YPD (1% yeast extract, 2% peptone, 2% glucose) at 30ºC for 24 h, collected by centrifugation at 4000 rpm for 5 min, washed once with sterile water and resuspended in a known volume of sherry wine. The numbers of total and viable cells were counted under an epifluorescent microscope according to the method previously described [11]. Vessels containing 18 L of sherry wine, sterilized by filtration, were inoculated with 1×10^6 viable cells/mL in order to form the *flor* velum biofilm. The vessel mouth was plugged with a mosquito net and vessels were kept in the dark at 18ºC. The specific surface (surface/v ratio) in these vessels was 0.052/cm. When the alcohol content dropped below 13.5%, a volume of 6 L of sherry wine (1/3 vol) was removed and then replaced with an equal volume of fresh sherry wine containing distilled alcohol in order to adjust the ethanol concentration in the vessel to 15.5% and to refresh the aging wine. This sequence of operations imitates the drawing-off and refilling procedures of an industrial dynamic system for sherry wine aging. Seven of the pure cultures were grown for 180 days, while the culture of the VPDN strain was maintained for 310 days.

Analytical Parameters

Ethanol concentration, acetaldehyde concentration and volatile acidity were analyzed in each culture once a week. Ethanol was quantified according to the transflexion technique using the Infra-analyzer™ 450 system from Technicon™ (New York, USA), according to the manufacturer's instructions. Volatile acidity was determined by flow injection (FIA) in an Auto-analyzer™ II from Technicon™ (New York, USA), according to the method previously described by Owades and Dono [12], and acetaldehyde concentration was then calculated according to the procedure recommended by the OIV [13].

Table 1. Strains of *Saccharomyces Cerevisiae Flor* Yeast Used in This Study

Strains	Molecular patterns		Origin
	Karyotype[a]	mtDNA-RFLP[b]	
B10	I	A1H1R1	Sherry wine yeast strain isolated from Añada A [5]
BS13	I	A1H1R1	Sherry wine yeast strain isolated from Añada A [5]
BS24	I	A3H1R1	Sherry wine yeast strain isolated from Añada A [5]
CHS7	III	A2H2R2	Sherry wine yeast strain isolated from Añada B [5]
CHS24	III	A2H2R2	Sherry wine yeast strain isolated from 2nd criadera [4]
B17	III	A2H2R2	Sherry wine yeast strain isolated from Añada B [5]
CHS15	XXII	A2H3R3	Sherry wine yeast strain isolated from 5th criadera [4]
VPDN	XIII	A5H5R6	Wine yeast strain isolated from the velum developed by 11763 (obtained from the CECT) in sherry wine pure culture under laboratory conditions (University of Cadiz)

[a]Electrophoretic karyotypes were obtained by PFGE. [b]Different mitochondrial RFLP patterns were obtained by using endonucleases *Alu*I, (patterns A1, A2, A3 and A5); *Hinf*I (patterns H1, H2, H3 and H5) and *Rsa*I (patterns R1, R2, R3 and R6)

RESULTS AND DISCUSSION

Analysis of the Biofilm Behaviour of the *Flor* Yeast Strains

For the study of enological behaviour, we used the strains listed in Table **1**, which had previously been characterized by molecular techniques, to enable a microbiological control procedure to be implemented. The activity of the biofilm growing on the surface of the aging wine during the period of study was monitored by recording two aging indexes: acetaldehyde concentration and volatile acidity (expressed as the concentration of acetic acid). In general, the cultures exhibited a pattern of irregular increase in acetaldehyde concentration (with alternating periods of accumulation and consumption), and a decrease in volatile acidity (Table **2**). These variations indicated differences in the behaviour of the biofilm covering the aging wine. In the group of *flor* yeast strains with chromosomal DNA and mtDNA pattern 'III, A2H2R2', the strains CHS7 and B17 showed similar and lower acetaldehyde concentration than CHS24 in pure culture (Table **2**). This difference could be associated with the origin of the strains, as has been shown in other studies [14], because CHS24 was from a dynamic system (2nd criadera, see Table **1**) and CHS7 and B17 were isolated from a static system (Añada B) in which the wine was younger. However, a mechanism for adaptive evolution in *flor* yeast has previously been reported [15]. It is possible that these two strains are less well adapted than CHS24 to biological aging conditions and, for this reason, their behaviour is different and they produce a lower acetaldehyde concentration. The strains B10 and BS13 with molecular pattern 'I, A1H1R1' and the same origin (Añada A) also showed similar behaviour, and the acetaldehyde concentration in the wine was higher than with the strains with patterns 'III, A2H2R2' isolated from Añada B. It should be noted that the acetaldehyde concentration in the wine with BS24 was lower than in those with B10 and BS13 (Table **2**), which had the same electrophoretic karyotype but a different *mt*DNA-RFLP pattern.

Although the strain CHS15 was different in respect of both its molecular pattern and origin, in comparison with the other strains isolated from sherry wines, that strain generated about 300 mg/L of acetaldehyde. This was similar to the value corresponding to BS13. Finally, the VPDN biofilm produced the most acetaldehyde in the wine. Only minor differences in volatile acidity were found between the different pure cultures (Table **2**). Such differences did not produce any noticeable effect on the properties of the aged wine. Among all the yeast biofilms, average values of volatile acidity were less than 0.18 g/L, which has been described as a very low (acceptable) value in sherry wines [16].

Kinetics of Biofilm Growth and Acetaldehyde Accumulation in the Wine

For the eight pure cultures assayed, the process of development of the *flor* velum biofilm was similar, with two main stages being observed: phase I or *velum formation* (PhI) and phase II or *velum maintenance* (PhII). PhI started when the first groups of yeast cells appeared on the surface of the wine (a few days after the inoculation was performed) and lasted until the whole surface was covered by a thin film of yeast cells. This phase was characterized by exponential cell growth, during which the cell viability remained higher than 90% in most of the cultures, and its duration, ranging from 9 to 21 days, was found to be strain-dependent (Table **3**). Then a rapid transition to PhII took place: the yeast biofilm was still growing but suddenly became thicker. PhII could be considered a stationary phase in which the thick biofilm covering the whole surface of the wine was composed of cells fewer than 10% of which were viable, and that situation remained unchanged. The periodic operations of drawing-off and refilling fresh wine, carried out in imitation of the industrial procedures, produced breaks in the velum, causing mixing and dilution of the compounds, such as acetaldehyde, in the aging wine. We performed these enological practices repeatedly during the experiments, and each time it led to a short-term transition to a renewed phase I (secondary phase I, 2nd PhI), which was followed by a renewed phase II (2nd PhII). This general pattern of evolution of acetaldehyde concentration can be observed for the case of strain BS24 in Fig. (**1**).

Table 2. Acetaldehyde Concentration and Volatile Acidity in Eight Different Pure Cultures During Biological Aging. Average, Standard Deviation, Maximum and Minimum Values for the Period of Study are Shown

Culture	Acetaldehyde (mg/L)			Volatile Acidity (g/L)			Total Measures[a]
	Av.± SD	Max	Min	Av.± SD	Max	Min	
B17	113 ± 11	130	100	0.08 ± 0.03	0.17	0.05	27
CHS7	140 ± 27	187	99	0.12 ± 0.06	0.23	0.06	25
BS24	176 ± 45	238	95	0.10 ± 0.04	0.19	0.06	24
CHS24	203 ± 35	246	162	0.10 ± 0.04	0.17	0.05	25
B10	233 ± 72	470	80	0.17 ± 0.06	0.35	0.06	24
BS13	281 ± 65	387	217	0.15 ± 0.08	0.30	0.07	25
CHS15	306 ± 70	472	234	0.10 ± 0.03	0.16	0.06	26
VPDN	479 ± 147	740	157	0.16 ± 0.05	0.30	0.08	37

[a]Measures were made each seven days throughout the process

Table 3. Average Rates of Acetaldehyde Accumulation During Different Phases of the Biofilm Growth, Described in the Text. The Average Duration (in Days) of These Phases in Each Culture is Also Presented

	Av. Rate of Acetaldehyde Accumulation (mg/L per day)		Av. Duration of Phase (days)	
Culture	Phase I[a]	Phase II	PhaseI[b]	Phase II
B10	17.5 (30.0)	-17.0	8.8 (13)	13.0
BS13	13.1 (25.9)	-9.6	15.2 (12)	14.0
BS24	13.4 (18.3)	-5.1	8.7 (7)	15.4
CHS15	22.2 (36.0)	-13.6	15.0 (11)	17.5
CHS7	11.7 (22.2)	-5.0	10.8 (5)	17.5
CHS24	14.3 (33.0)	-6.0	25.0 (5)	9.3
B17	5.9 (13.5)	-3.2	13.4 (4)	9.0
VPDN	8.9 (9.4)	[c]	20.8 (53)	[c]

[a]Specific average rates were computed including successive PhI's. The accumulation of the acetaldehyde in each PhI was calculated measuring the increase from each operation of drawing-off and refilling until the value drops, and taking into account the days after. Values in brackets correspond to the acetaldehyde accumulation during the initial phase of velum formation (PhI). [b]The duration of the initial PhI and each successive PhI was determined based on both acetaldehyde concentrations and observation of the biofilms. The higher values in brackets correspond to the initial phase of velum formation (1st PhI), whereas the average duration of phase was computed including the initial and successive PhI's. In the case of VPDN, the periods of acetaldehyde accumulation were only interrupted by the operations of drawing-off and refilling with new wine. [c]The biofilm of the VPDN strain remained in the phase of velum formation (PhI) during the whole period of study.

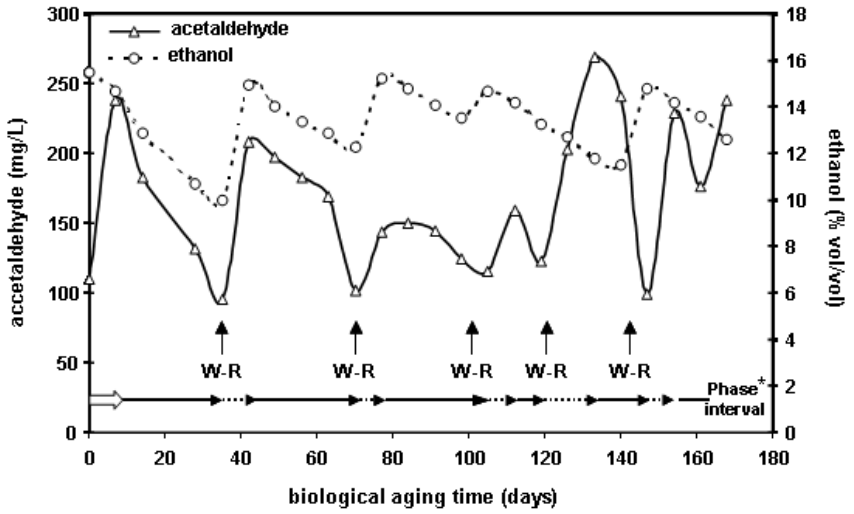

Fig. (1). Evolution of ethanol and acetaldehyde concentrations in the BS24 culture. Times of the operations of drawing-off and refilling (W-R) with fresh fortified wine are indicated by vertical arrows. *Duration of the phases is represented by different horizontal arrows: ⇒, PhI; ·····>, 2nd PhI induced by operations of drawing-off and refilling; →, PhII or 2nd PhII. Figure shows that Ph I and 2nd PhI corresponded to periods of acetaldehyde accumulation, whereas PhII and 2nd PhII corresponded to acetaldehyde depletion.

However, each culture displayed a relatively high rate of acetaldehyde accumulation during the initial PhI (Table **3**), compared with the rate occurring during successive 2nd PhI's, which was lower.

In contrast, during PhII, acetaldehyde no longer accumulated in the wine but was depleted at a strain-dependent rate (Table **3**). The decrease in acetaldehyde concentration observed in PhII is in agreement with previous results, indicating that once the thicker velum is formed, cell biosynthesis stops [17]. Hence the concentration of acetaldehyde decreases until a refilling with new fortified wine leads to a temporary transition to a 2nd PhI.

It should also be noted that the VPDN strain behaved differently from the other strains during aging. We observed that it formed a thin biofilm which did not completely cover the surface of the wine, and this partial biofilm remained in PhI. Moreover, the biofilm in the 1st PhI I took longer to form than in the other strains - about 53 days (Table **3**). This culture showed an almost constant rate of acetaldehyde accumulation (around 9 mg/L per day, Table **3**), although a high average concentration of 479 mg/L was reached (Table **2**). After the first period of velum formation, the proportion of viable cells decreased slowly to values of about 40% of

the total, whereas the cell viability of other strains showed a faster and steeper reduction (to values of less than 10%).

The CHS15 culture also showed acetaldehyde accumulation at a relatively high rate (36 mg/L per day) during the first 11 days. The transition to PhII then occurred, and the acetaldehyde concentration decreased (to an average rate of 13.6 mg/L per day) until the next operation of drawing-off and refilling with fresh wine induced a 2nd PhI. The average duration of the repeated 2nd PhI's was 15 days, during which acetaldehyde accumulation increased again to an average rate of 22.2 mg/L per day (Table **3**). The high level of acetaldehyde accumulation recorded in wine aged by CHS15 (Table **2**) is possibly because CHS15 was observed to be able to release a relatively large amount of acetaldehyde into the wine during PhI. Nevertheless, the acetaldehyde accumulation in the CHS15 culture was not as high as that in the VPDN culture, because the CHS15 culture remained in PhII for a long period, during which time the cell viability was extremely low (below 10%) and acetaldehyde was being depleted.

The cell viability of the VPDN culture during the sherry wine aging process was higher and more constant compared with the other strains of this study (data not shown), which may be associated with the strain's general capacity for producing higher acetaldehyde concentrations, as observed by other researchers [18, 19]. Therefore, the high level of acetaldehyde in the VPDN culture could be due to its capacity to resist the high acetaldehyde concentration in the assay conditions. Its cells remained for a longer time in PhI, in a slow but exponential growth phase, releasing acetaldehyde at a low but constant rate, and that explains the high acetaldehyde concentration recorded in the wine aged by this strain (Table **2**).

In the cultures of the CHS15, B10 and BS13 strains, the time needed for the biofilm to cover the surface of the wine after the inoculation coincided with the duration of PhI. Strains B10 and BS13 behaved similarly in the development of the biofilm, also accumulating high concentrations of acetaldehyde (Table **3**). However, the formation of the biofilm took about 14 days in BS24, CHS7, CHS24 and B17 whereas acetaldehyde accumulation stopped several days before this was completed (Table **3**). It has been reported that acetaldehyde exerts deleterious effects on yeast cells when its concentration reaches a threshold, which is variable according to the strain [19, 20]. Presumably, action to mix the culture and so homogenize the acetaldehyde concentration in the whole culture volume would be advantageous. This hypothesis is supported by the fact that, in these cultures, the duration of the 2nd PhI induced by a refilling operation, which produced some mixing, was longer than that of the 1st PhI (Table **3**).

Design of Strategies to Improve Wine Aging

It seems reasonable that the differences in enological performance, particularly in growth kinetics and acetaldehyde productivity, shown in the present results can be associated with the different genetically-characterized strains of the *S. cerevisiae flor* yeast. These strains were isolated from fino-type sherry wines, in which acetaldehyde is considered to be the most influential component in the organoleptic properties of the finished wine [16]. Thus, when a sherry winery is seeking to improve the biological aging process, it is necessary to select those yeast strains that generate larger amounts of acetaldehyde and that form the initial biofilm more rapidly after inoculation, partly to accelerate the aging biological process and partly to enhance the organoleptic properties. With these objectives, the VPDN strain is likely to be a candidate in a selection program for a particular industrial environment and, in turn, the newly-selected VPDN strain could be used as inoculum. This strain, which shows unique chromosomal and *mt*DNA patterns, produces the continuous accumulation of more-than-sufficient amounts of acetaldehyde and therefore its inoculation in the blending stages would accelerate the biological aging of the wine. Nevertheless, the biofilm activity of the VPDN pure culture is too low to cover the whole wine surface, in an industrial environment; this inadequacy could lead to excessive oxidation of the wine. Moreover, its rate of acetaldehyde accumulation during PhI was very low. Taking all these factors into consideration, the VPDN strain should be used in combination with other yeast strains, to compensate for those disadvantages of the VPDN strain. The utilisation of wine yeast in mixed cultures is a practice that has been proposed for inoculating alcoholic fermentations [21]. The genetic manipulation of *flor* yeast to improve sherry wine production has also been proposed [14], but the current regulations in the Jerez region do not permit the use of genetically modified yeasts.

The inoculation of the mixed cultures is therefore suggested. For example, the CHS15, B10 or BS13 strains could be used initially, not only to accumulate acetaldehyde at a high rate during PhI but also to establish a complete biofilm across the whole wine surface. Prior to the transition to PhII, the wine could be inoculated with the VPDN strain, to continue the accumulation of acetaldehyde so that the wine is fully aged more rapidly, with optimum properties. Thus, further studies on the behaviour of different *flor* yeast strains in mixed cultures are considered of interest and of industrial relevance.

CONCLUSIONS

In this study, we have found that *flor* yeast strains of *S. cerevisiae* with identical chromosomal and mitochondrial DNA patterns, and the same origin, showed a more closely-related enological behaviour, but no correspondence was found between a particular molecular pattern and physiological behaviour in, for example, the concentration of acetaldehyde produced in the wine. The kinetics of biofilm growth was similar for the strains assayed. We observed that each pure culture displayed specifically a high rate of acetaldehyde accumulation during PhI and a decrease in this parameter in PhII. No correspondence was observed between rates of acetaldehyde accumulation and consumption, and the molecular pattern of the strain. However, taking into account other parameters including the time (in days) taken for the initial biofilm to form, the strains with identical pattern, such as B10 and BS13 (pattern 'I, A1H1R1') and CH7, CHS24 and B17 (pattern 'III, A2H2R2') do show similar behaviour. The approach described in this article is important for the entire Enology sector, because there is little information available on the design of strategies for using selected *flor* yeast strains to improve wine aging on the industrial scale.

Those strains that generate a high concentration of acetaldehyde and form a complete biofilm more rapidly will be good candidates for further assays to be carried out in industrial conditions.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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