Direct Enumeration and Isolation of Wine Yeasts from Grape Surfaces

ALESSANDRO MARTINI[1]*, MAURIZIO CIANI[2], and GLORIA SCORZETTI[3]

Recent ecological surveys indicate that the wine yeast *Saccharomyces cerevisiae* may be isolated with extreme difficulty from natural substrates, such as vineyard soil or the surface of mature grapes, conventionally believed to be its elected habitat. Conversely, it is amply demonstrated that its preferential location as the only fermenting colonizer is the surfaces of the wineries. Non-conventional methods for the separation, isolation, and enumeration of yeast cells from mature grapes of different varieties produced additional and unequivocal evidence on the numerical inconsistency of *S. cerevisiae* cells in nature. The taking over of musts by the natural yeast flora was also followed in microfermentations.

KEY WORDS: *Saccharomyces cerevisiae*, ecology, grape surface, must, natural fermentation

Pasteur's early interpretation (14), later confirmed by Martinand and Rietsch (10), Boutroux (4), and Berlese (3) proclaims that the conversion of must into wine is a spontaneous process brought about by the resident yeast flora of the grape surface.

A redundant series of ensuing ecological surveys reviewed by Kunkee and Amerine in 1970 (8), categorically promulgated the theorem that cells from the wine yeast *Saccharomyces cerevisiae* are omnipresent in nature, with a tangible propensity for vineyard soils where they prosper on grapes fallen from vines. Vineyard soil was assigned the role of winter quarter in the cycle of *S. cerevisiae* in nature and postulated to represent the ultimate pool of yeasts with favorable technological properties selected through the years for the specific task of fermenting the musts of the particular grape variety growing in that specific site. It is most unfortunate, however, that not even the faintest evidence was provided in favor of the cyclic nature of the circulation model originally propounded by Pasteur. In other words, it is still unknown how and when the yeast cells that multiplied and fermented the must in the winery return to the vineyard soil, survive through the winter and are ready to start again the cycle when grapes are ripe.

In addition, in the majority of the above surveys, the actual isolation step was always preceded by an enrichment culture in sterile grape must or by an "autoenrichment" when the course of a natural fermentation was followed in freshly pressed grapes. On that account, at least two, highly selective factors of must as a culture medium [sugar concentration always >16% to 18% and the anaerobiosis condition] enact a strong selective pressure in favor of those yeasts capable of fermenting highly concentrated sugary liquids.

[1,2,3]Industrial Yeasts Collection DBVPG, University of Perugia, Dipartimento di Biologia Vegetale, Sezione di Microbiologia Applicata, Borgo 20 Giugno 74, I-06122 Perugia, Italy.

*Corresponding author.

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For this reason, those studies of the past essentially based on enrichment (or autoenrichment) in grape must consistently yielded biased results in favor of the only and sole species with strongly fermentative catabolism, namely *S. cerevisiae* . Accordingly, the well known and currently accepted abundance in nature of *S. cerevisiae* is the unique and specific outcome of a methodological artifice.

Recent ecological evidence based on direct isolation procedures, excluding any enrichment effect [for details see review by Martini (11)], clearly and definitely indicate that *S. cerevisiae* is isolated with extreme difficulty [less than 10 colony forming units (cfu) per square cm of fruit surface or gram of soil] from conventional habitats such as vineyard soil or the surface of ripe grapes or any other sugary fruit; while it is almost the sole fermenting species colonizing massively the surfaces of the winery as demonstrated by Peynaud and Domercq (15), Rosini (16,17), and Rosini *et al.*, (19).

In order to produce additional evidence on the actual yeast flora of the grape surface at vintage time, we carried out a survey on the presence of yeasts on grapes from ten separated parcels of the experimental vineyard of the School of Agriculture of our university, each cultivated with a different variety of *Vitis vinifera* L. Various methodological approaches were used for the separation, isolation and enumeration of yeast cells. In addition, the course of the taking over of musts by natural yeasts was followed in microfermentations.

Materials and Methods

Samples: Parts of grape bunches from different vine plants (3 to 4 kg) were aseptically collected in plastic autoclavable bags immediately before harvesting time (first week of October 1995). Single grapes were aseptically separated from a single bunch and placed individually in a sterile glass tube.

The experimental vineyard, included within the farm of the School of Agriculture of the University of Perugia, is located at 300 m altitude on a hillside south of the town of Perugia, in a grape growing area. The following grape varieties were considered: Cabernet Sauvignon, Ciliegiolo, Grechetto, Merlot, Monte-

pulciano, Sangiovese, Trebbiano Umbro, and Trebbiano Toscano.

Cell enumeration on the surface of grapes: Each grape berry was transferred into a 25- × 80-mm glass tube containing 10 mL of sterile water and individually submitted to the following sequence of pre-isolation treatments, whose overall efficacy in dislodging yeast cells from natural surfaces had been previously tested in a series of ecological surveys from this laboratory (12,18).

Jet-streaming: A simple plastic 5-mL syringe equipped with a AIG 40.7 needle was used to generate a high pressure jet of liquid. The operation was repeated five to six times by pumping back sprayed water into the syringe.

Fast shaking: The jet-washed grape was then shaken on a Vortex Super Mixer for 60 seconds.

Sonication: The sample was finally sonicated for 30 seconds in a MSE Scientific Instruments Ultrasonic Disintegrator (Crawley, Sussex, United Kingdom) operating at 35 W/cm^2 of acoustic energy at probe tip.

Cell counts were carried out in duplicate using the "serial dilution method." One milliliter of the cell suspension obtained after the three treatments described above, was inoculated into 9 mL of steam-sterilized (15 min at 100°C) grape must in 10- × 60-mm glass tube. Grape must tubes were serially inoculated until a 10^{-5} dilution. After five to seven days of incubation at 25°C, tubes with growth were microscopically inspected, and the shape of enriched cells was recorded with a rough estimate of the ratio of elliptical (E) to apiculate (A) forms.

Cell enumeration on grape must: The content of each bag of grapes was aseptically pressed and reciprocally shaken (180 rpm) for 15 to 20 minutes in order to improve the release of yeast cells from skins.

Total cell counts were then performed in duplicate by the pour plate method on two media: Unipath WL-Nutrient Agar and Unipath Lysine Agar (Unipath, Basingstoke, Hampshire, England), which is unable to support the growth of *S. cerevisiae* (9). Counts were made after seven days incubation at 25°C.

Microfermentations in natural conditions: Seventy milliliters of pressed and shaken grape must were aseptically transferred into a 100-mL Erlenmeyer flask sterilely stoppered with a glass fermentation trap containing sulfuric acid to allow only CO_2 to escape the system (5). The weight loss of the fermentation vessel from escaping CO_2 was followed for several days until the end of the fermentation. Direct isolations were performed on WL-Nutrient Agar after four days of fermentation at 25°C and at the end of the fermentation. Colonies were selected according to their macro- and micromorphological aspect, and isolated proportionally to their frequencies.

Classification procedures: The method suggested in the taxonomic monograph of Kreger van Rij (7) was followed for the identification of isolates; assimilation tests were performed using the ID32C Identification System for Yeasts (Bio-Mérieux, France).

Analytical determinations: Fermented musts were filtered through membranes (GF/C, Whatman International, Maidstone, England) and conserved at -20°C until analysis.

Ethanol (as % v/v) was estimated by gas-chromatography (1). Volatile acidity (as g/L of acetic acid) was determined by steam distillation (6).

Results and Discussion

Results of cell counts will be reported and discussed in relation to the two procedures employed. The information from microfermentations under natural conditions will be discussed separately.

Direct cell enumeration on grape surfaces: It is known from the redundant series of ecological surveys on the yeast flora associated with winemaking [reviewed by Kunkee and Amerine (8)] that the species *Kloeckera apiculata* is always and massively present during the first phase of natural grape must fermentations, while *S. cerevisiae* takes over after a few days and concludes the process; the large majority of the remaining yeast species isolated from fermenting musts (*ca* 150) is considered occasionally present.

Accordingly, we decided to concentrate our attention only on the two species always associated with winemaking, because they are capable of enduring the highly selective conditions of grape must as a culture medium. Grape must is a differential growth medium because of its low pH (3.5), which prevents the growth of most bacteria, and because of its high sugar content (often >20%, w/v), which only permits the growth of those yeasts able to ferment the substrate; and among the fermenting ones, only of those capable of producing and tolerating high concentrations of ethanol.

A feature often affecting results in studies of microbial ecology is the presence of many breaks and organic secretions on plant surfaces that may firmly entrap cells or microcolonies in minute and deep channels and/or in gummy, waxy, and mucous materials. As previously demonstrated in this laboratory (12,18), the application of vigorous and disruptive pre-isolation treatments to the separation of microbial cells from their natural habitats allows the recovery of a greater variety of yeast species as well as causing an increase in total cell counts of several order of magnitude. In particular, the dislodging efficiency of these more aggressive procedures was tested with several sugary fruits and other natural substrates (12) and grapes (18). Accordingly, counts were performed on cell suspensions obtained by treating each berry with a sequence of three aggressive and cell-dislodging procedures.

For actual cell counts, we used a method that consists of making decimal serial dilutions in a liquid medium such as must and recording the tubes showing positive microbial growth. Since dilutions exhibiting no growth presumably failed to receive even a single yeast cell, the "most probable number" of viable cells may be

Table 1. Enumeration by "serial dilution in grape juice (autoenrichment)" of fermenting yeast cells on the surface of single berries from different cultivars of *Vitis vinifera* L.

Samples →	Grape one*				Grape two*				Grape three*			
Dilutions →	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Cabernet	—	—	—	—	E	—	—	—	E	—	—	—
Ciliegiolo	A	A	—	—	A	—	—	—	A	—	—	—
Grechetto	A	—	—	—	A	—	—	—	A	—	—	—
Merlot	A	—	—	—	A	—	—	—	A	A	A	—
Montepulciano	A + E	A + E	—	—	A + E	A	A	—	A	—	—	—
Sangiovese	A	—	—	—	A	—	—	—	A	—	—	—
Trebbiano Umbro	A + E	A	—	—	A + E	—	—	—	A + E	A	A	—
Trebbiano Toscano	A	A	—	—	A	A	—	—	A	A	–	–

*Yeast growth: — = absent; A = lemon-shaped (apiculate) cells; E = round to oval (elliptical) cells.

statistically inferred from the distribution of growth (MPN presumptive test).

Results of cell counts reported in Table 1 unequivocally demonstrate that the yeast flora colonizing the surface of ripe grapes is numerically limited. Let us bear in mind that the succession of aggressive treatments on single grapes yielded altogether a 10-mL suspension of dislodged cells and that decimal dilutions were carried through with 1 mL. Only on three grapes out of 24, were more than 1000 cfu (10^{-4} dilution) present; on five samples more than 100; while in the majority of cases the average number of yeast cells colonizing the berry surface appeared to be between 10 and 100 per grape. Assuming that each berry is a sphere of 1 mL volume, weighing 1 g, its surface is around 6 cm^2. Accordingly, values of about 10^5 cfu per mL of must reported in the literature for grapes from New Zealand (13), France (2), and Italy (18) correspond to an average yeast content ranging from 1×10^4 to 1×10^5 cells per cm^2. Figures found in this investigation for the entire mature berries are considerably lower, with most of the samples colonized by less than 100 cells per cm^2 and scarcely any carrying a few thousand colony forming units.

In order to correlate data from microscopic inspection on the distribution of elliptical (E) and lemon-shaped (A) forms with actual identification of the corresponding species, streak cultures on YEPG were performed from the 1×10^{-2} dilutions. Colonies were selected and isolated according to their morphological aspect (shape, color, edge, and surface appearance) and after microscopical inspection; identification of isolates was obtained from the examination of microscopic characters and results of assimilation tests carried out with ID32C. The taxonomic composition of the yeast flora of grapes confirms and supports all the observations of the above mentioned ecological surveys: the "apiculate" yeast *Kl. apiculata* is the species numerically prevailing on sugary fruit surfaces, while round to oval-shaped cells from non-fermenting and film-forming species (*Pichia*) or low ethanol-tolerant forms (*Metschinikowia pulcherrima*) are often present. Apparently, members of the high ethanol-tolerant species *S. cerevisiae* are very difficult to isolate (3 berries out of 30 and only after enrichment).

Direct cell enumeration in aseptically pressed grapes: Counts were carried out in duplicate on juice from aseptically pressed grapes by the spread plating method on two different media. Nutrient Agar is a general purposes medium, normally used for the enumeration of fungi from natural environments, while Lysine Agar is often used in the brewing industry for growing non-*Saccharomyces*, contaminating yeasts (9).

The purpose of our test was the indirect estimation of the approximate percentage of the total yeast population represented by cells of *S. cerevisiae* unable to grow in the presence of lysine.

Table 2. Direct cell counts on musts from freshly pressed grapes of different cultivars and composition of the yeast flora of grape surfaces.

Must samples from pressed grapes of the cultivar	Colony forming units/mL [× 1000]		Identification of selected colonies on counting plates*			
	Nutrient agar	Lysine agar	*Metsch. pulcherrima*	*Kl. apiculata*	*S. cerevisiae*	Film-forming yeasts
Cabernet	2885	3090	8	3	—	—
Ciliegiolo	437	781	7	—	—	7
Grechetto	66	114	—	4	—	2
Merlot	10	10	5	4	—	1
Montepulciano	230	835	4	3	—	2
Sangiovese	547	1460	2	3	—	11
Trebbiano Umbro	132	190	—	5	—	9
Trebbiano Tosc.	8550	6715	3	—	—	9

*Colonies were selected according to their macro- and micromorphological aspect and isolated proportionally to their frequencies.

Results appearing in Table 2 show that the various grape varieties are characterized by a numerically different yeast flora, ranging from 1×10^4 to 1×10^6 cells per mL. Lack of significant differences between counts on the two culture media excludes the presence of *S. cerevisiae* cells; concurrently, the counts on lysine agar appear consistently higher for all grape cultivars.

Direct isolation culture on nutrient agar plates originated an overall situation paralleling that found in the previously reported identification tests: the same low-ethanol fermenting and film-forming species as well as the complete absence of high ethanol fermenters of the genus *Saccharomyces*.

Natural microfermentations: The evolution of the yeast flora during fermentations proceeding in natural conditions, without addition of selected starters for wine making, has been the object of innumerable, redundant investigations since the beginning of this century (8). The term "natural" is conventionally used in wine microbiology to designate a grape must fermentation initiated by the yeast flora "naturally" present in the environment, while a "guided" process requires the use of a selected yeast as fermentation starter. In freshly pressed grapes several microbial groups can be found besides the predominating yeast flora. Bacteria, filamentous fungi, and sporadically, protozoa are normally present and may take over in musts obtained by hail-damaged grapes. Nevertheless, it has been known for a long time (10) that *Kl. apiculata* cells initiate the process in the majority of cases. *S. cerevisiae* cells are so scarcely represented on grape surfaces (less than 10 per cm^2) that their growth in musts becomes apparent only after four to five days of fermentation, when the initial numerical advantage of apiculate yeasts (near 2×10^5 cells per cm^2 of grapes) is counterbalanced by their inability to tolerate the ethanol concentration accumulated by their own anaerobic metabolism. By virtue of their faster growth rate on highly concentrated sugary juices and much higher ethanol tolerance, elliptical cells rapidly multiply, completely taking over the substrate in a few hours and bringing the fermentation process to completion.

The above scenario has been largely verified and corroborated by results of hundreds of natural fermentations carried out in all of the vine-growing areas of the world (8). Exceptions to the rule, such as stuck fermentations, growth of unusual microorganisms, unacceptable characters of fermentations and/or a complete absence of growth in some cases, have often been observed in newly established vineyards, especially those of geographical areas never cultivated with grapes before and supposedly characterized by soils not yet harboring specialized, fermenting yeast cells.

The evolution of the yeast flora during the natural microfermentations carried out in this research is illustrated in Table 3. According to predictions, after five days all samples exhibited a clear prevalence of apiculate forms, an occasional presence of film forming yeasts, and a complete absence of *S. cerevisiae* cells. After 10 days, at the end of the fermentation period, viable *Kl. apiculata* cells were isolated only in two samples, while bacteria had taken over in nine out of 10 musts. The most striking result was the consistent absence of *S. cerevisiae* throughout the entire series of tests.

As a matter of fact, this was to be expected as the obvious consequence of previous results (Tables 1 and 2) from both cell enumeration tests performed on the same samples. Apparently, no cells of *S. cerevisiae* were present in the 3 to 4 kg of grapes collected or the exceedingly few possibly available were not adequate to be developed by the enrichment culture in the highly selective grape must medium.

The fact that bacterial cells occupy the fermenting liquids is a direct consequence of the absence of the only yeast capable of carrying to completion the transformation of sugar into ethanol beyond the upper tolerance limit of *Kl. apiculata* , about 3.5% to 4.0% (v/v). Under this condition, bacteria grow either on the residual sugar or on the ethanol formed during the first step of natural fermentations.

The complete deviation from the norm observed in the microbial succession, necessarily resulted in repercussions on the chemical composition of fermentation products. As seen in Table 4, results were highly anomalous in nine samples. Only the Grechetto cultivar exhibited overall analytical results comparable with those of wine, even though the amount of acetic acid formed was unacceptably high.. From Table 3, it can be seen that it was possible to isolate *S. cerevisiae* only during the last stage of the Grechetto cultivar micro-fermentation. The concomitant presence of bacteria also explains the acetic acid content of near 4.0 g/L observed in this sample (Table 4).

Table 3. Evolution of the yeast flora during natural microfermentations carried out with juice from aseptically pressed musts from grapes of different cultivars.

Must samples from pressed grapes of the cultivar	Species isolated after 5 days of fermentation at 25°C			Species isolated after 10 days at 25°C at the end of fermentation		
	Kl. apiculata	film-forming yeasts	*S. cerevisiae*	*Kl. apiculata*	Other micro-organisms	*S. cerevisiae*
Cabernet	prevails	yes	absent	absent	bacteria	absent
Ciliegiolo	prevails	no	absent	few cells	bacteria	absent
Grechetto	prevails	no	absent	few cells	bacteria	yes
Merlot	prevails	no	absent	absent	film yeasts	absent
Montepulciano	prevails	no	absent	absent	bacteria	absent
Sangiovese	prevails	no	absent	absent	bacteria	absent
Trebbiano Umbro	prevails	yes	absent	absent	bacteria	absent
Trebbiano Tosc.	prevails	yes	absent	absent	bacteria	absent

Table 4. Analytical results of natural microfermentations carried out with juices from aseptically pressed grapes of different cultivars.

Cultivar	Initial sugar (% w/v)	Residual sugar (% w/v)	Ethanol (% v/v)	Acetic acid (g/L)
Cabernet	14.8	6.80	absent	12.0
Ciliegiolo	19.8	8.80	absent	25.5
Grechetto	19.7	0.2	11.7	3.9
Merlot	16.3	6.65	absent	14.3
Montepulciano	14.3	6.90	0.1	16.7
Sangiovese	18.0	9.00	absent	29.7
Trebbiano Umbro	8.33	5.81	absent	15.2
Trebbiano Toscano	18.1	8.50	absent	15.1

In the remaining nine tests, the massive presence of apiculate yeasts at the end of the first fermentation phase (5 days), accompanied by the observed degradation of consistent portions of the available sugar, is an indirect evidence of ethanol formation (theoretical ethyl alcohol contents from 2.1% to 6.6% in volume may be calculated taking into account a 0.6 yield coefficient). Concurrently, after 10 days of fermentation, ethanol was completely absent (Table 4) while acetic acid reached concentrations from 10- to 25-times higher than the accepted upper limit (6). At the same time, microbiological analyses demonstrated that bacterial cells were the predominating microorganisms at the end of the process.

It seems logical to conclude that, in the absence of viable cells of the high-ethanol-tolerant species *S. cerevisiae*, the ethanol formed by the early activity of *Kl. apiculata* was used as a carbon and energy source by bacteria naturally present in musts. The high concentrations of acetic acid observed indicate a possible role of species of the genus *Acetobacter* in the utilization of the ethanol originally formed.

Conclusions

The results presented in this paper, together with additional evidence from other investigations (11), allow an explanation of the presence and circulation of the species *S. cerevisiae* that appears *de facto* more scientifically established than the array of irrational and misleading data of the past. The new scenario may be summarized by the following points: *(i)* cells of *S. cerevisiae* are rarely isolated from natural surfaces, including grapes or vineyard soil; *(ii)* when present, their number never exceeds 10 colony forming units per cm^2; *(iii) ca* 80% of the yeast species colonizing the surfaces of wineries belong to the species *S. cerevisiae* (15,17,19); *(iv)* preliminary evidence (17) indicates that the natural fermentation of musts is carried out by the winery resident yeast flora; *(v)* the fermentation of musts from grapes aseptically pressed in the laboratory and never exposed to the atmosphere of an established winery is often anomalous; *(vi)* it is likely that the resident flora competes with those *S. cerevisiae* cultures inoculated as selected starters for winemaking, although actual experimental evidence is still lacking.

The above summarized ecological evidence reinvigorates and rehabilitates an old, wishful belief dating back to the turn of this century, when wine microbiologists were unsuccessfully searching for the local, individual, specific fermenting yeast flora, superselected after years of adaptation to each microclimatic area. We now know that their efforts were necessarily bound to failure because the objects of the selection are so scarcely represented in natural environments that any likelihood of cell reproduction is automatically excluded and consequently any possible genetical modification of enological characters.

We have plenty of evidence (15,17,19) that the winery environment is always colonized by a great many cells of *S. cerevisiae* that go through generations and generations at each vintage. This provides, theoretically and practically, the amount of variability necessary for the selection of a winery-specific strain. A selective pressure on the *S. cerevisiae* population of the winery may be working through conventional limiting factors such as ethanol concentration in favor of high ethanol producing strains, sugar concentration in favor of strains capable of fermenting in adverse osmotic conditions, or high concentrations of SO_2 in favor of strains resistant to its action. It is not easy, however, to conceive and scientifically justify the presence of a selective pressure causing the enrichment of strains capable of producing specific volatile compounds that characterize organoleptically the local wine, because such a property cannot possibly endow the strain possessing it with a clear advantage over the others.

In spite of the above conceptual limitation, there is already some preliminary evidence (19; unpublished data, 1996) showing that the strains of *S. cerevisiae* isolated from the winery environment possess technological properties on the average comparable with those of commercial selected starters. It is our hope that this conclusion will give rise to additional investigations on the indigenous yeast flora of wineries from different geographic areas.

One additional scope of these last considerations is to draw attention to the technological potentialities of the presence of specific metabolic abilities possibly producing distinct flavors, correlating with characters associated to local wines.

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