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ORIGINAL PAPER

A low-cost procedure for production of fresh autochthonous wine yeast

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Abstract A low-cost procedure was designed for easy and rapid response-on-demand production of fresh wine yeast for local wine-making. The pilot plant produced fresh yeast culture concentrate with good microbial quality and excellent oenological properties from four selected wine yeasts. The best production yields were obtained using 2% sugar beet molasses and a working culture volume of less than 60% of the fermenter capacity. The yeast yield using 2% sugar grape juice was low and had poor cell viability after freeze storage, although the resulting yeast would be directly available for use in the winery. The performance of these yeasts in commercial wineries was excellent; they dominated must fermentation and improved its kinetics, as well as improving the physicochemical parameters and the organoleptic quality of red and white wines.

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Abbreviations

OD 600	Optical density at 600 nm
T15	Time needed to ferment 15% of the total sugars present in the must
T100	Time needed to ferment 100% of the total sugars
cyh	Cycloheximide
smr	Sulfometuron
rhod	Rhodamine 6G
CFU	Colony-forming units

Introduction

The use of selected *Saccharomyces* yeasts for wine-making has clear advantages over the traditional spontaneous fermentation. Usually, the final product is of a better quality than the wine produced by traditional spontaneous fermentation [16, 17, 43, 45]. Recent years have seen an increase in the use of new autochthonous or local selected yeasts to control must fermentation. Selected local yeasts are believed to be much more effective than foreign selected yeasts because they are presumed to be more competitive from being better acclimated to the local environmental conditions. Therefore, they should be better able to dominate the fermentation and become the most important biological agent responsible for the wine-making process. Moreover, selection of the appropriate local yeasts assures the maintenance of the typical sensory properties of the wines produced in any given region [11, 26, 34].

To ensure the effectiveness of the must's inoculation with costly selected yeast, monitoring of the inoculated

strain is required to find out its degree of dominance during fermentation. For this purpose, techniques are needed to differentiate the inoculated strain from the wild yeasts in the must. Several procedures based on molecular polymorphisms have been used to solve this problem [3, 9, 18, 24, 25, 28, 29, 36, 47], but unfortunately most are expensive, complex and time consuming for most commercial wineries. As an alternative, some fast, reliable and economic methods involving natural wine yeast mutants with specific genetic markers have been developed to monitor must-inoculated yeast through fermentation [1, 2, 31]. These methods are suitable for industrial applications, as will be shown in this work.

The quality of commercial wine yeast is determined by its oenological properties and other parameters such as storage, stability, osmotolerance, freeze-thaw resistance and drying/rehydration resistance. While yeasts are marketed in different formats, such as fresh liquid yeast culture, solid culture on agar, pressed yeast, lyophilized or active dry yeast, it is this last form that is most sold worldwide, mainly because it is of low volume and can be stored for longer than 1 year, which reduces costs. However, dry yeast needs to be reconditioned, and the recovered yeast's vitality depends on storage conditions and the lipid composition of the cell membrane [39].

In *S. cerevisiae* production, high sugar concentrations and high specific growth rates trigger alcoholic fermentation, even under fully aerobic conditions [10, 32]. Alcoholic fermentation during the industrial production of wine yeast is highly undesirable, as it reduces the biomass yield from the carbohydrate feedstock. In industrial fed-batch cultivation, the actual growth rate is controlled primarily by the feed rate of the carbohydrate feedstock [6, 40]. Generally, an initial exponential feed phase is followed by phases with constant and declining feed rates [14]. The aerobic conditions result in low protein and high carbohydrate contents, which enhance the stability of the yeast that is desired for its storage. On the contrary, anaerobic growth increases the yeast's enzymatic content, improving its fermentative capacity [21]. Process optimization during fed-batch cultivation has been based on strain selection and optimization of environmental conditions (e.g., pH, temperature, aeration rate and feed profiles of sugar, nitrogen and phosphorus) [8, 21, 42].

Although many different carbohydrate feedstocks can be used for yeast production, most companies use cane or beet molasses, which are low-cost by-products from sucrose production companies. Molasses are dense and viscous liquids, with a high amount of solid residues (68–75%) and 48–52% of sucrose [7, 30]. The composition is highly variable depending on the sucrose refining procedure and on the year's weather conditions [21], which can affect the yield and quality of the yeast produced. In modern fed-batch processes for yeast production, the feed of molasses is under strict control based on the automatic measurement of ethanol traces in the fermenter exhaust gas. Using this system, the biomass yield is near the theoretical maximum obtainable, although it usually results in low growth rates and longer yeast production times. Usual industrial processes involve fermenter volumes of 50,000–350,000 l or greater. The first two inoculum stages usually involve relatively low-volume aseptic fermentations, while later inoculum and production stages are usually done in large-volume fermenters under non-aseptic conditions. All these fermentation processes are usually very expensive because of the intensive use of technological devices and the need for skilled workers. However, the production of great amounts of a single yeast strain for several days is usually very profitable, even with the inclusion of the downstream processes of yeast washing, concentration, drying and packaging. Unfortunately, the above production setup is not very profitable for medium to low amounts of very often different autochthonous yeast strains for local wine-making. Local wine yeast could be produced on demand in a production plant close to the wineries just before or during the harvest season and sold as fresh liquid concentrate culture. This is cheaper to produce than active dry yeast, and the yeast is ready for must inoculation without the need of previous reconditioning.

The aim of the present study was to design a low-cost plant for easy and rapid response-on-demand production of fresh wine yeast for local wine-making. We analyzed the microbial quality and the oenological properties of the fresh yeast culture concentrate produced with several selected strains, their degree of dominance in commercial wineries and their usefulness for elaboration of good quality wine.

Materials and methods

Yeast strains and analytical culture media

The yeast strains used in this work are summarized in Table 1. JP88, E7AR1, SMR16-5A and RhodM2H5-6D are autochthonous K2-killer prototrophic and homothallic *S. cerevisiae* wine yeasts previously isolated from Spanish wineries, selected [43] and genetically improved [37, 38] for wine-making. Standard culture media were used for yeast growth and phenotype tests [48]. YEPD agar (yeast extract peptone dextrose agar) contained 1% Bacto-yeast extract, 2% Bacto-peptone, 2% glucose and 2% Bacto-agar. YEPD + cyh is YEPD agar supplemented with cycloheximide (cyh) (prepared as a 1% solution in 96% ethanol) to a final concentration of 2 μg/ml [31]. SD + smr is standard SD agar (synthetic dextrose agar) supplemented with sulfometuron (smr) (prepared in a 1% dimethyl sulfoxide

Table 1 Wine yeast strains used for yeast production

Strain	Genotype (phenotype)	Origin
JP88	*MATa/α HO/HO cyh^S/cyh^S* [k2$^+$]	J.A. Regodón[a]
E7AR1	*MATa/α HO/HO CYH^R/cyh^S* (CYH2^R) [k2$^+$]	F. Pérez-Nevado[b]
SMR16-5A	*MATa/α HO/HO SMR^R/smr^S* (SMR^R) [k2$^+$]	J. Ambrona[c]
RhodM2H5-6D	*MATa/α HO/HO PDR5/pdr5* (RHOD^{PC}) [k2$^+$]	J. Ambrona[c]

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[b] F. Pérez-Nevado, Departamento de Producción Animal y Ciencia de los Alimentos, Área de Nutrición y Bromatología, Universidad Extremadura, Badajoz, Spain. E7AR1 is a cycloheximide-resistant (*cyh2*^{R}) wine yeast from the 7AR hybrid [37, 38] sold by Heral SL (Almendralejo, Spain)

[c] J. Ambrona, Departamento de Ciencias Biomédicas, Área de Microbiología, Universidad de Extremadura, Badajoz, Spain. SMR16-5A is a sulfometuron-resistant (*SMR*^{R}) wine yeast [1]. RhodM2H5-6D is a rhodamine-pink cycloheximide-resistant (*RHOD*^{PC}) wine yeast [2]

solution) to a final concentration of 100 µg/ml [1]. YEPD + rhod is YEPD agar supplemented with rhodamine 6G (rhod) (prepared as a 1% solution in water) to a final concentration of 5 µg/ml [2]. The drugs cyh, smr and rhod were added to the medium just before this was poured into Petri dishes.

Contamination of the yeast produced by bacteria, molds and wild yeasts was analyzed by plating appropriately diluted culture samples in duplicate on YEPD for wild yeasts and mold counts (incubated at 25°C for 48–72 h), VRBA (violet red bile agar) for coliform count (incubated at 37°C for 24–48 h), VRBG (violet red bile glucose agar) for enterobacteria counts (incubated at 37°C for 24–48 h), MRS agar (man rogosa sharp agar) for *lactobacilli* counts (incubated at 30°C for 48 h in an automatic CO_2 incubator, NuAire, USA), M17-agar for *Streptococcus* counts (overlaid with the same medium and incubated at 37°C for 24–48 h) and GYC (glucose yeast extract calcium carbonate agar) for acetic acid bacteria counts (incubated at 30°C for 48–72 h).

Setup of the yeast production plant

The plant was designed for rapid on-demand production of fresh yeast for quality wine elaboration in the surrounding wineries. A 24-h production time was set (from 10:00 am to 10:00 am of the next day) to get a 10× fresh yeast concentrate (after culture centrifugation) with 4–8 × 10^9 viable yeast cells/ml ready for grape must inoculation. Two low-cost stainless steel vessels were used as fermenters, one with 100 l of full capacity (46-cm diameter × 61-cm height) used for the 25 and 50 l of yeast culture productions and another with 350-l capacity (65-cm diameter × 1.2-m height) for the 100-, 200- and 300-l productions (Table 2). The first one is currently available on the market for less than €300 (Superfuxtinox mod. MILANO 18/10, Milan, Italy), and the second was specially made by a local company (Transfinox, Villafranca de los Barros, Spain) for less than €2,000. Each fermenter has the minimum of equipment required so as to keep cost low: an aseptic inoculation pipe, a drain point, an additional sampling point (in the 350-l vessel only) and a sterile air line with a perforated ring sparger (holes of 2-mm diameter) located at the bottom of the vessel. Before each production run, all the equipment was carefully washed first with 2% NaOH, then with water and detergent, rinsed with water and steam sterilised with water vapor at 100°C (Karcher). The tubes and valves were washed with 96% ethanol. Each pure strain culture to be used as a yeast inoculum in the 25-, 50- and 75-l production runs was prepared in sterile YEPD broth (2.5–7.5 l) in an adjoining laboratory under aseptic conditions. The original starter culture came from frozen yeast suspensions (−80°C in 15% glycerol). The inocula for the 100-, 200- and 250-l production runs were obtained from previous 50-l runs in the 100-l fermenter. The yeast inocula (roughly 5 × 10^7 cells/ml based on the theoretical final volume) were added to the starting base media (for the composition, see below). Then, the carbohydrate feedstock (molasses or grape juice) was fed-batch added with a peristaltic pump (LKB P1 of Pharmacia-Biotech or 313U of Watson-Marlow) to the growing yeast culture during the first 20 h following one of two different programs depending on the culture working volume using a Micro DCU-300 System (Braun Biotech International; Fig. 1, Table 2), maintaining at all times the actual sugar concentration below 0.005% to avoid sugar fermentation and ethanol production. Culture aeration and agitation were done with a sterile, filtered (0.45-µm diameter) air flow of 3 vvm (gas volume per reactor volume per minute), using a regenerative blower (GAST IDEX Corporation, Benton Harbor, MI). Yeast production runs were done at room temperature under non-aseptic conditions, starting at around 10 days before vintage in August and continuing until the end of September. Foam production was avoided when required (mostly just during the first 3 h of yeast growth) by the addition of 1–2 ml of Antifoam 64 (Sigma-Aldrich). After completion of the carbon source addition, the culture was maintained with aeration for 4 h to allow yeast maturation. This phase is to expend all the fermentable sugars and favors glycogen and trehalose synthesis by the yeasts, which will increase yeast stability during storage [5, 21, 49]. Finally, the yeast culture was concentrated ten times using a nozzle-type centrifuge (Alfa Laval SLDX 209-30B, Alfa Laval Corporate AB, Lund, Sweden), washed with sterile water, and the centrifuged cells were resuspended in sterile water.

Table 2 Working conditions for yeast production

Culture volume (l)[a]	Fermenter capacity (l)[b]	Diluted feedstock volume (l)	Feedstock addition program
25	100	10	I
50	100	10	I
75	100	10	I
100	350	20	II
200	350	20	II
250	350	20	II

[a] Theoretical working culture volume, without considering liquid evaporation loss

[b] Maximum capacity of the fermenter

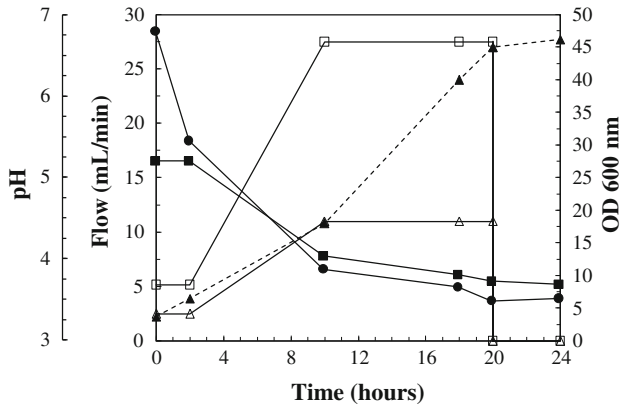

Fig. 1 Feedstock addition programs for yeast production, evolution of the culture pH during yeast production and culture growth kinetics of a good-yield production; *opened triangle* program I, *opened square* program II, *filled triangle* OD 600 of an E7AR1 production, *filled circle* pH of molasses culture medium, *filled square* pH of grape juice culture medium

Although such parameters as oxygen consumption, oxygen concentration in the culture, air flow, sugars in the medium and ammonia are usually controlled in commercial yeast production to improve yield [21, 22], these parameters were not monitored to avoid increasing the production cost involved in the related equipment and the need for qualified employees.

Starting base media and carbohydrate feedstock preparation

The molasses starting base medium contained 2 g/l yeast extract, 0.75 g/l $(NH_4)_3PO_4$, 1.6 g/l $MgSO_4{\cdot}7H_2O$, 5 mg/l thiamine, 0.1 mg/l biotin and 1 mg/l calcium pantothenate. Its pH was 6.6. The grape juice starting base medium contained 2 g/l yeast extract, 10 g/l $(NH_4)_3PO_4$, 0.6 g/l $MgSO_4{\cdot}7H_2O$ and 3 g/l $(NH_4)_2SO_4$, 5 mg/l thiamine, 0.1 mg/l biotin and 1 mg/l calcium pantothenate. The pH was adjusted to 5.2 with 85% orthophosphoric acid. The molasses feedstock was beet molasses (387.6 g/l sucrose, 54.2 g/l glucose, 48.5 g/l fructose) appropriately diluted with chlorinated water depending on the culture working volume and adjusted to pH 3.5 with 35% HCl. The grape juice feedstock was commercial sulfited grape must (230 g/l glucose + fructose, pH 3) appropriately diluted with chlorinated water.

Culture conditions, growth measurements and freeze storage

Starter cultures for biomass production were grown in YEPD broth at 30°C on a rotary shaker at 200 rpm for 24 h. Cell growth was measured by determining the optical density at 600 nm using a spectrophotometer. To determine culture dry weight, culture samples (10 ml) were filtered through pre-weighed 47-mm membrane filters (Millipore Corp., 0.45-µm pore size). The filters were washed with distilled water twice and then dried at 100°C for at least 16 h to constant weight. The determinations were performed in duplicate and varied by less than 2%. For the evaluation of cell viability after freeze storage, aliquots of the ten-fold-concentrated yeast cultures were mixed with sterile glycerol to 15% and stored at −20°C. After freeze storage, the aliquots were left for 15 h at 4°C in a cold room before dilution with sterile water and plating on YEPD agar. The plates were incubated at 30°C, and the colony-forming units (CFU) were determined after 48–72 h.

Vinification trials

For the winery vinification trials, the molasses-produced yeasts (10× concentrate) were inoculated in 1,000–2,000-l stainless steel tanks containing fresh white must (mix of varieties *Cayetana*, *Pardina*, *Eva* and *Montúa*, 21.25 ± 1.1 mean ºBrix, mean pH 3.54 ± 0.18 after addition of tartaric acid, 50 mg/l of SO_2, and clarified by settling for 18 h at 12°C) or de-stemmed crushed grapes (*Tempranillo*, 22.39 ± 1.6 mean ºBrix, pH 3.62 ± 0.14 after addition of tartaric acid, 70 mg/l of SO_2) to a final concentration of 0.5–1 × 10^7 cells/ml. An uninoculated control (spontaneous fermentation) was performed in parallel for each vinification series. A total of eight independent series were done in four different wineries, two in each winery—one series of red wines and one of white wine. As a result, 40 wines were obtained, 8 controls and 32 inoculated with the 4 yeasts produced (8 with each). The vinification process was conducted at 18°C for white wine and at 24°C for red wine. The must density and the ºBrix were monitored every day. The tanks were capped hermetically when reducing sugars reached around 1% to avoid oxidation problems. At the end of fermentation, the settled solids were discarded. An 800-ml centrifuged sample of each wine was taken for the assays. The uncentrifuged wines were stored at 4°C.

After 50 days following the end of fermentation, settled solids were again discarded, and the wines were returned to storage at 4°C. At 85 days, settled solids were discarded once more, and the wines were bottled. At 105 days following the end of fermentation, the sensory characteristics (flavor, color and odor) of the wines were tested by a panel of 12 experts. Wines were presented in clear, tulip-shaped wine glasses covered with glass Petri dishes. A sample of 50–70 ml of wine was poured into each glass immediately before evaluation by each judge. The temperature of the samples was from 10 to 13°C for white wines and from 16 to 18°C for red wines. The judges scored the quality of the wines on a six-point scale (0 = very poor, 1 = deficient, 2 = acceptable, 3 = good, 4 = very good and 5 = excellent). The maximum score possible (60 points) was considered 100% of preference. Hydrogen sulfide odor was determined by the judges on a 30-unit (U) scale (0 = no odor, 10 = low odor, 20 = high odor, 30 = very high odor).

Determination of the proportion of inoculated yeasts in the vinification trials

The percentage of genetically marked yeasts (*cyh2*^{R}, *SMR*^{R} and *RHOD*^{PC} mutants) was determined by the replica-plating method [1, 2, 31]. Samples from fermenting musts in the tumultuous stage and at the end of fermentation were diluted and plated onto YEPD-agar to obtain 200–300 colonies per plate. The detection of the *cyh2*^{R}, *SMR*^{R} and *RHOD*^{PC} yeasts was accomplished by replica-plating these plates to either YEPD + cyh (2 μg/ml), SD + smr (100 μg/ml) or YEPD + rhod (5 μg/ml) plates using sterile velvets and then to other plates of YEPD agar to detect wild yeasts sensitive to cyh or smr. To check these genetic detection methods, the percentage of inoculated yeasts was also determined by analyzing the mtDNA restriction pattern [1, 2, 31, 35] in one sample of each must vinification trial at the tumultuous fermentation stage. Purified mtDNA was digested with *RsaI*. The fragments were separated in 0.5 × TBE-0.8% agarose gels for 75–90 min and were visualized on a UV transilluminator after ethidium bromide staining. The percentage of JP88 yeasts was determined by analyzing the mtDNA restriction pattern of 50 YEPD-agar isolated colonies from each sample.

The proportions of non-*Saccharomyces* and bacteria were determined at the beginning of must fermentation because they usually fall off rapidly, becoming very low-frequency microorganisms with respect to *Saccharomyces* yeasts. *Saccharomyces* "sensu stricto" [29] colonies are easy to distinguish from the other yeast species present in fermenting grape must by their aspect in YEPD agar (white or cream color, buttery, smooth, circular and prominent) and by their ability to sporulate, producing typical asci (tetrads) [20].

Analytical methods

Density, ºBrix, pH, total acidity, volatile acid, reducing sugars, alcohol and lactic acid were determined according to the EEC-recommended methods [13]. Malic acid was determined using the EC-recommended method [12]. Major volatile compounds and polyols were quantified by gas chromatography [27]. T15 is the time needed to ferment 15% of the total sugars present in the must, and T100 is the time needed to ferment 100% of the total sugars [38]. The polyphenol index was determined as previously described [44].

Statistical analysis

Data were analyzed for statistical significance by the Krustal-Wallis and Mann-Whitney non-parametric tests or ANOVA parametric test. A 5% probability level ($P = 0.05$) was used to accept or reject the null hypothesis. All the statistical analyses were performed with the software package SPSS version 12.0 for Windows (Chicago, IL).

Results and discussion

Environmental parameters during the fed-batch yeast production

A total of 54 yeast production runs were made, 42 with molasses and 12 with grape juice. The theoretical working volume was the sum of those of the starting base medium, the yeast inoculum and the carbohydrate feedstock. The actual final recovered volume was roughly 86–97% of the theoretical final working volume because of the liquid evaporation during the yeast culture aeration. The working room temperature varied from 22 ± 3°C during the night to 30 ± 2°C during the day, and yeast culture temperature varied from 25.8 ± 0.3°C to 29.3 ± 0.3°C, very close to the optimum proposed for yeast production of 28.5–30°C [21]. After inoculation, the initial optical density at 600 nm (OD 600) of the culture was around 4, reaching around 46 by the end of the best production processes, 4–6 × 10^{8} viable yeast cells/ml (as referred to the final theoretical volume before culture concentration; Fig. 1). The initial pH of the starting base media was 6.6 ± 0.2 and 5.2 ± 0.1 for molasses and grape must, respectively, which are higher values than those recommended for optimal yeast production, which are from 4 to 5.5 [21]. However, after 10 h of addition of pH-adjusted molasses or grape juice, the pH decreased to 3.9 ± 0.1 and 4.0 ± 0.2, respectively, equal to or lower than the pH 4 recommended to avoid microbial growth of undesirable microorganisms [21].

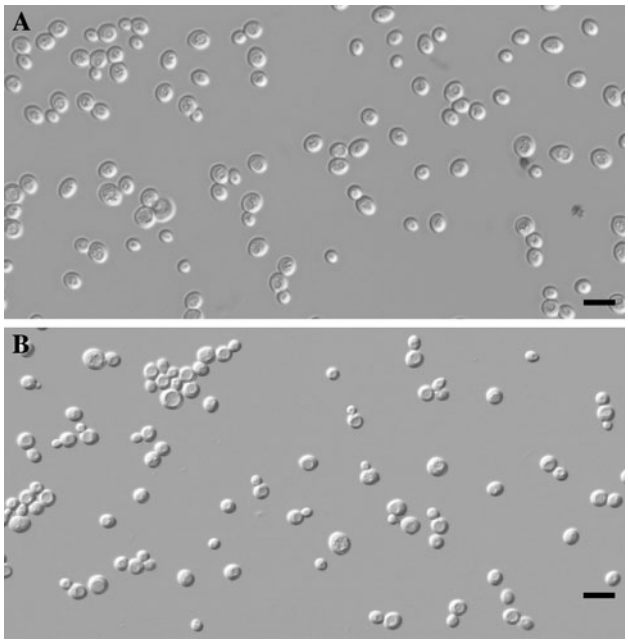

Fig. 2 Nomarski micrographs (×400) for JP88 yeast produced in the 350-l fermenter (200 l of culture, 2% sugars) with molasses (**a**) or grape must (**b**). The micrographs were taken after the 24-h production run. The *scale bar* represents 10 μm

Microbial quality of the produced yeast

Microscopy revealed that most of the yeasts produced were pure cultures, with homogeneous cell size and morphology for molasses productions and more variable cell size for grape juice productions (Fig. 2), with more than 96% of live cells (as measured by methylene blue stain, data not shown). The cell size variability obtained in the grape juice productions is due to a high proportion of yeast still budding, and there are more young yeasts than in the molasses productions. No contamination by wild yeasts, acetic bacteria, coliforms, enterobacteria or filamentous fungi was observed in most productions. Only one production was contaminated with 1×10^7 cells/ml of *Streptococcus* sp. (detected in M17 culture media) and another with 4.2×10^7 cells/ml of *Lactobacillus* sp. (detected in MRS media), both using the yeast SMR16-5A. In both of these productions, the predicted pH control failed to yield a final pH below 4.2, and this could have favored the growth of these contaminating lactic acid bacteria. Microbial contamination is frequent in industrial yeast production because, given the great size of the fermenters, it is difficult to maintain sterile conditions [4, 41]. Most contamination sources are well known—such as the feedstock, failure of sanitary conditions, deficient equipment cleansing, or the surrounding air [19]. In sum, however, our simple experimental design allowed the production of wine yeast with a great proportion of viable cells and good sanitary quality as long as the pH was properly maintained below 4.

Influence of the carbon source on yeast production

The yield of fed-batch yeast production with beet molasses depended on the amount of total sugar added during yeast growth (1, 1.5, 2 or 2.5%, expressed as a percentage of the theoretical final volume of the yeast culture). The yeast yield increased from 19 ± 2 g/l using 1% sugars (0.52 g/g sugars) to 31 ± 1 g/l using 2% of sugar (0.44 g/g sugars) and drastically decreased to 8 ± 3 g/l when sugars were further increased to 2.5% (0.1 g/g sugars), an even lower yield than when using 1% sugars (Table 3). This is explained by the yeast's change from respiratory to fermentative metabolism (Crabtree effect) when increasing the amount of added sugars above 2% under our working conditions [21, 50].

The yeast yield using grape juice at 2% sugar concentration (15.5 ± 0.8 g/l, 0.22 g/g sugars) was significantly lower than the yield with 2% molasses (29.6 ± 2.2 g/l, 0.42 g/g sugars; Table 4). Also, the yeast cell size in the grape juice production was more variable and generally smaller than in the molasses productions (Fig. 2). It seems therefore that nutritionally the grape juice used for these productions was not as good a feedstock as the beet molasses. However, despite the lower yeast yield, the yeast produced with grape juice is almost ready for winery must inoculation, in contrast to molasses-produced yeast that requires a prior wash to remove browning and off-flavor

Table 3 Influence of molasses-sugar concentration on the yeast production yield

Yield	Percentage of added sugars[a]				P[b]
	1	1.5	2	2.5	
Grams of dry yeast/l	19 ± 2B	23 ± 2.6B	31 ± 1C	8 ± 3A	***
Grams of dry yeast/grams of sugar	0.52 ± 0.02B	0.44 ± 0.06B	0.44 ± 0.04B	0.10 ± 0.05A`	***

[a] Percentage of added sugars relative to the theoretical final culture volume

[b] *P*-values obtained with the ANOVA test (*** $P \leq 0.001$). The data are the mean ± standard errors of 18 independent experiments (200-l productions), 9 with E7AR1 (2 with each sugar concentration except 3 with 2%) and 9 with RodM2H5-6D (2 with each sugar concentration except 3 with 2%). Different letters (*A*, *B* and *C*) mean significantly different groups found with the Duncan test at $P < 0.05$

compounds. The amount of viable cells per gram of dry yeast was greater for must than for molasses productions (1.2×10^{10} vs. 1.1×10^{10} CFU/g, although the differences were not statistically significant, Table 4). If this difference is real, it could partially obviate its lower yeast weight yield when inoculating wine fermentations at the winery. Therefore, yeast production with grape must needs to be considered as an alternative option that could further simplify and cost-reduce the process, although yeast stability should also be considered if cold (4°C) or freeze storage (−20°C) is required. Indeed, for cold storage, after 1 month, the molasses-produced yeasts maintained 98.7% of cell viability and the grape must-produced yeasts 94% (Table 4), while after 3 months, the viability was highly variable, decreasing to 50–94% (data not shown). For freeze storage, after 3 months, the molasses-produced yeasts maintained 85.3% of cell viability, while the grape-must-produced yeasts presented only 13.2% viability (Table 4). The reason that the molasses-produced yeasts are more resistant to freeze storage than must-produced yeasts is probably because they grew faster and had less fermentation activity by the end of the yeast production process, making them more resistant to freeze-thaw stress [33, 46, 49]. Given these results, cold-storage periods shorter than 1 month should be recommended for molasses- or grape must-produced yeast, while molasses production is advised instead of grape must production if yeast freeze-storage is required before winery applications.

Influence of fermenter size and culture volume on yeast production

To allow the adaptation of yeast production size to winery demand, we analyzed the effect of culture volume on yeast yield in the 100- and 350-l fermenters, always working below 76% of fermenter capacity to avoid foam problems. All production runs were done with 2% sugar-molasses. In each fermenter, the highest yeast yield was obtained with the lowest culture volume (36 ± 3 g/l culture for 25 l in the 100-l fermenter and 33.8 ± 3 g/l for 100 l in the 350-l fermenter, Table 5). Increasing the working culture volume significantly decreased the yeast yield in both fermenters. This is probably because of oxygen limitation, although the amount of incoming air was presumably enough to fully support aerobic yeast growth—3 vvm, above the recommended 0.5 vvm [21, 50]. Probably the air distribution in the culture was not optimal and could be improved to increase yield, although this would increase the equipment cost. Despite this, most yeast production runs can be considered satisfactory except those of the 75- and 250-l cultures, whose yields were below 20 g/l culture (Table 5). Therefore, working culture volume below roughly 60% of the fermenter capacity can be recommended to obtain good yields under our working conditions. Production with relative volumes greater than 60% is not feasible because the increased production volume does not compensate for the decrease in yield.

Influence of the wine yeast strain on the yeast production

The yeast productions were done with four wine yeast strains—JP88, E7AR1, SMR16-5A and RodM2H5-6D. The yeast yield varied depending on the strain, from 28 ± 1.8 g/l (0.4 g/g sugars) for SMR16-5A to 32.2 ± 3.1 g/l (0.46 g/g sugars) for E7AR1, although the differences were not statistically significant (Table 6). These results were somewhat expected, as all four yeast strains are genetically related and were isolated from the same wine production area and selected for wine-making following the same oenological selection criteria. It is conceivable that these apparent biomass yield differences may become greater if one were to work with an optimized (and far more expensive) production procedure, in which any slightly relevant genetic difference among these four yeast strains could

Table 4 Influence of the sugar feedstock on the yeast production yield and yeast viability after cold- and freeze-storage

Yield	Feedstock		P[a]
	Molasses	Grape juice	
Grams of dry yeast/l	29.6 ± 2.2	15.5 ± 0.8	***
Grams of dry yeast/grams of sugar	0.42 ± 0.05	0.22 ± 0.02	***
CFU/ml culture	$3.4 \times 10^8 \pm 4.6 \times 10^7$	$1.9 \times 10^8 \pm 1.8 \times 10^7$	**
CFU/g dry yeast	$1.1 \times 10^{10} \pm 1.3 \times 10^9$	$1.2 \times 10^{10} \pm 1.2 \times 10^9$	ns
% viability after cold-storage[b]	98.7 ± 3.8	94 ± 4.1	ns
% viability after freeze-storage[c]	85.3	13.2	*

[a] P-values obtained with the Mann-Whitney non-parametric test (*** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$); *ns* not significant ($P \geq 0.05$)

[b] After 30 days at 4°C

[c] After 90 days at −20°C in 15% glycerol. The data are the mean ± standard errors of 24 independent experiments (200-l productions with 2% sugar concentration), 6 with each yeast strain (three with each feedstock)

Table 5 Influence of culture volume on the yeast production yield

Yield	100-l fermenter			350-l fermenter		
	25 l (25)	50 l (50)	75 l (75)	100 l (33)	200 l (67)	250 l (71)
Grams of dry yeast/l	36 ± 3	32.6 ± 1.5	19 ± 0.4	33.8 ± 3	31.2 ± 2	17 ± 1.5
Grams of dry yeast/grams of sugar	0.51 ± 0.06	0.47 ± 0.04	0.27 ± 0.06	0.48 ± 0.08	0.44 ± 0.04	0.17 ± 0.02

The data are the mean ± standard errors of 28 independent productions of E7AR1 and RodM2H5-6D with 2% sugar-molasses, 14 with each strain (2 of 25 l, 3 of 50 l, 2 of 75 l, 2 of 100 l, 3 of 200 l and 2 of 250 l). The percentage of the fermenter capacity used for the yeast culture is in parentheses. All differences between the data for each fermenter size were statistically significant (non-parametric Kruskal-Wallis test, $P \leq 0.05$)

Table 6 Yeast yield for the fed-bacth molasses production of four wine yeast strains

Yield	Yeast strain				P^a
	JP88	E7AR1	SMR16-5A	RodM2H5-6D	
Grams of dry yeast/l	30.1 ± 3	32.2 ± 3.1	28 ± 1.8	31.5 ± 2.2	ns
Grams of dry yeast/grams of sugar	0.43 ± 0.06	0.46 ± 0.08	0.4 ± 0.05	0.45 ± 0.06	ns

[a] *P*-values obtained by Kruskal-Wallis test. *ns* not significant ($P \geq 0.05$). The data are the mean values and standard errors of 24 independent experiments with 2% sugar-molasses, 12 of 50 l (3 with each strain) and 12 of 200 l (3 with each strain)

result in different growth efficiencies. These yields were greater than those reported previously for production of *S. cerevisiae* with virgin grape marc, 19.8–23.5 g/l [23], but were similar and compared well with other yeasts produced with a mix of molasses and cheese whey, 32.3–35 g/l [15]. In sum, therefore, the yeast yields for all four tested wine yeast strains were similar and reasonably good as compared with other yeast strain productions.

The produced yeast's fermentation performance in the wineries

Winery vinifications were performed with fresh white must (mix of different varieties, see Materials and Methods) or red grapes (*Tempranillo*) using the four yeast strains produced with molasses. An uninoculated control (spontaneous fermentation) was done in parallel for each vinification series. A total of 8 different vinifications series were performed, resulting in 40 wines. Statistically significant differences were found in the must fermentations and the resulting wines elaborated with the produced yeast strains and those of the uninoculated vinifications (Tables 7 and 8), as is coherent with previous studies [16, 43, 45]. All the inoculated yeasts showed similar must fermentation parameters (T15 and T100), with the uninoculated control fermentations being much slower. The initial yeast population in the inoculated must fermentations was much higher than in the uninoculated control fermentations. In the inoculated fermentation, the yeast population increased rapidly by roughly one order of magnitude after 20–24 h of inoculation, this increase being less in the white musts than the red, probably because of the lower temperature of the white relative to the red wine fermentation (data not shown).

The dominance of all the inoculated yeasts in tumultuous fermentation (measured by using the genetic marker assays or the mtDNA restriction pattern analysis) ranged from 85 to 100%, mostly from 97 to 100% (Tables 7 and 8). Roughly the same values were found in the subsequent end of the fermentation stage of each inoculated vinification. Additionally, 20 YEPD-plate-isolated colonies from each sample of the E7AR1-, SMR16-5A- and RodM2H5-6D-inoculated and the uninoculated vinifications were subjected to mtDNA restriction pattern analysis. All the yeast colonies from the inoculated vinifications bearing the genetic markers (*cyh2*^{R}, *SMR*^{R} or *RHOD*^{PC}) had the same mtDNA restriction pattern as the original inoculated strain. No colonies of the inoculated yeasts were detected in the uninoculated controls (data not shown).

The amount of non-*Saccharomyces* yeasts and bacteria was higher in the uninoculated controls than in the inoculated ones, although the differences were not significant for the amount of bacteria in the red wines. Therefore, inoculation with the produced yeast decreased the relative presence of undesirable wild microorganisms during the must fermentation. Total acidity was higher in the inoculated wines than in the controls, and pH and volatile acidity were lower. The amount of reducing sugars was lower in the inoculated wines, which is consistent with a better end of fermentation, i.e., a lower T100 (Tables 7 and 8). These results are important for wine-making because a low amount of reducing sugars and high total acidity improve the microbial stability of the wines during

Table 7 Must fermentation parameters and wine analysis results of 20 independent winery vinifications made with mixed white grapes. ANOVA was used to study the effect of the yeast strain produced on wine-making

Parameter	Inoculated yeast					P^a
	None	JP88	E7AR1	SMR16-5A	RodM2H5-6D	
T15 (days)	3.85 ± 0.5B	2.29 ± 0.8A	1.9 ± 0.5A	1.6 ± 0.2A	1.95 ± 0.4A	*
T100 (days)	15.7 ± 1.7B	10.7 ± 0.9A	9.97 ± 0.98A	11.2 ± 0.7A	10.9 ± 1.4A	*
Initial yeasts/ml ($\times 10^5$)	0.2 ± 0.1A	64 ± 11AB	55 ± 7AB	80 ± 12B	71 ± 16AB	*
Dominance of yeast (%)[b]	0 ± 0A	85 ± 9.6B	97 ± 1.3B	98 ± 1.1B	99 ± 0.6B	***
Non-*Saccharomyces* (%)[c]	30.4 ± 9.5B	1.6 ± 1.6A	1.6 ± 1.5A	1.2 ± 1A	5 ± 5A	***
Bacteria (%)[d]	4.1 ± 3B	0.01 ± 0.01A	0.02 ± 0.02A	0.002 ± 0.002A	0 ± 0A	*
Alcohol (% v/v)	12.9 ± 0.4	13.3 ± 0.2	13.2 ± 0.2	13.1 ± 0.2	13 ± 0.3	ns
pH	3.41 ± 0.05	3.35 ± 0.04	3.38 ± 0.04	3.33 ± 0.04	3.32 ± 0.02	ns
Total acidity (g/l)	6.28 ± 0.12A	6.45 ± 0.26A	6.58 ± 0.51A	6.78 ± 0.29AB	7.71 ± 0.28B	*
Volatile acidity (g/l)	0.52 ± 0.08B	0.28 ± 0.07A	0.29 ± 0.03A	0.21 ± 0.020A	0.4 ± 0.07B	**
Reducing sugars (g/l)	1.47 ± 0.45B	0.35 ± 0.18A	0.38 ± 0.14A	0.37 ± 0.2A	0. 6 ± 0.21A	*
Malic acid (g/l)	1.22 ± 0.4	1.7 ± 0.4B	1.9 ± 0.27	1.6 ± 0.23	1.6 ± 0.19	ns
Lactic acid (g/l)	0.29 ± 0.16B	0.03 ± 0.03A	0.04 ± 0.035A	0.06 ± 0.034A	0.01 ± 0.006A	*
Ethyl acetate (mg/l)	42 ± 12	42 ± 16	52 ± 14	27 ± 13	39 ± 11	ns
Fusel alcohols (mg/l)	205 ± 31	217 ± 38	245 ± 26	254 ± 30	265 ± 25	ns
1-Propanol (mg/l)	20 ± 4	22 ± 4	25 ± 1	27 ± 3	30 ± 2	ns
Isobutanol (mg/l)	34 ± 5	34 ± 4	41 ± 3	34 ± 2	41 ± 1	ns
Amyl alcohols (mg/l)	151 ± 27	160 ± 35	179 ± 24	192 ± 29	195 ± 24	ns
H_2S (U)[e]	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1.4 ± 1.4	ns
Preference (%)	57.2 ± 3.4	59.8 ± 3.5	59.9 ± 2.4	60.3 ± 5	63.2 ± 2.8	ns

[a] *P*-values obtained by ANOVA for the wines made with each yeast. (*** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$). *ns* not significant ($P > 0.05$)

[b] Data relative to the total *S. cerevisiae* like CFUs measured in a sample from tumultuous fermentation (2–3 days after the inoculation time)

[c] Data relative to the total microorganism CFUs measured 1 day after the inoculation time

[d] Data relative to the total microorganism CFUs measured 1 day after the inoculation time

[e] *U* arbitrary units. The data are the mean values and standard errors of 20 independent experiments (4 uninoculated controls, 4 with JP88, 4 with E7AR1, 4 with SMR16-5A and 4 with RodM2H5-6D). Different letters (*A* and *B*) mean significantly different groups found with the Duncan test at $P < 0.05$

storage, and a low wine pH (3.3–3.5) improves the organoleptic perception.

The amount of malic acid was higher in the inoculated wines than in the control wines, and the amount of lactic acid was lower. This indicates the presence of some malolactic activity in the uninoculated wines, probably due to the contaminating wild microorganisms that were more frequent in the controls than in the inoculated wines.

The organoleptic quality (preference) was higher in the inoculated wines, although the differences were not significant for the white wines. There were no significant or relevant differences in the means of the rest of the analyzed parameters (alcohol, ethyl acetate, fusel alcohols, 1-propanol, isobutanol, amyl alcohols and H_2S; Tables 7 and 8).

These results indicate that the technological improvements usually obtained by an inoculating selected yeast strains during wine-making [1, 2, 43] are also achieved by using the wine yeasts produced in our low-cost fermentation plant.

Conclusions

Good-quality wine yeast can be produced in our low-cost production plant without any particular control of environmental conditions. No growth of undesirable microorganisms was observed as long as the pH was maintained below 4. The best production yields were obtained using beet molasses at 2% final sugar concentration and working with culture volumes below 60% of the fermenter capacity. No relevant differences were found when further decreasing the volume or using either a 100- or a 350-l fermenter. The yeast yield using 2% sugar grape juice was significantly lower than using 2% sugar molasses, and it had poorer cell viability after freeze storage. No yeast yield differences were found in the productions made with the four wine yeast strains (JP88, E7AR1, SMR165AR and Rod256D). The performance of these yeasts in the wineries was excellent relative to the uninoculated controls. They improved the must fermentation kinetics and decreased the amount of

Table 8 Must fermentation parameters and wine analysis results of 20 independent winery vinifications made with red *Tempranillo* grapes. ANOVA was used to study the effect of the produced yeast strain on wine-making

Parameter	Inoculated yeast					P^a
	None	JP88	E7AR1	SMR16-5A	RodM2H5-6D	
T15 (days)	4.4 ± 0.3B	2 ± 0.4A	1.7 ± 0.3A	1.8 ± 0.2A	1.8 ± 0.3A	***
T100 (days)	6.8 ± 0.2B	4.4 ± 0.6A	4.2 ± 0.3A	4.5 ± 0.4A	4.6 ± 0.5A	***
Initial yeasts/ml ($\times 10^5$)	0.3 ± 0.1A	85 ± 15B	90 ± 10B	77 ± 13B	82 ± 16B	*
Dominance of yeast (%)[b]	0 ± 0A	98 ± 1.5B	100 ± 0B	99.4 ± 0.4B	99.9 ± 0.1B	***
Non-*Saccharomyces* (%)[c]	66 ± 19B	2.9 ± 1.9A	2.5 ± 1.7A	1 ± 0.5A	1.6 ± 0.6A	***
Bacteria (%)[d]	1.32 ± 1.29	0 ± 0	0 ± 0	0 ± 0	0.01 ± 0.01	ns
Alcohol (% v/v)	138 ± 0.42	14.5 ± 0.42	14.3 ± 0.45	14.3 ± 0.33	14.2 ± 0.55	ns
pH	3.5 ± 0.04	3.5 ± 0.05	3. 5 ± 0.05	3.57 ± 0.05	3.58 ± 0.03	ns
Total acidity (g/l)	6.8 ± 0.52	7.1 ± 0.73	7.4 ± 0.67	7.21 ± 0.36	7.06 ± 0.82	ns
Volatile acidity (g/l)	0.25 ± 0.37	0.19 ± 0.18	0.2 ± 0.02	0.22 ± 0.02	0.26 ± 0.02	ns
Reducing sugars (g/l)	0.42 ± 0.15B	0.12 ± 0.08A	0.1 ± 0.06A	0.1 ± 0.06A	0.07 ± 0.04A	*
Malic acid (g/l)	1.7 ± 0.3	2 ± 0.2	2.3 ± 0.2	2 ± 0.2	2.1 ± 0.1	ns
Lactic acid (g/l)	0.38 ± 0.14B	0.11 ± 0.05A	0.07 ± 0.03A	0.14 ± 0.03A	0.1 ± 0.02A	*
Ethyl acetate (mg/l)	26 ± 10	23 ± 10	28 ± 11	20 ± 11	15 ± 7	ns
Fusel alcohols (mg/l)	332 ± 79	316 ± 63	273 ± 47	311 ± 47	270 ± 32	ns
1-Propanol (mg/l)	30 ± 7	36 ± 13	26 ± 9	45 ± 12	40±	ns
Isobutanol (mg/l)	48 ± 15	59 ± 21	47 ± 12	34 ± 5	47 ± 7	ns
Amyl alcohols (mg/l)	254 ± 62	221 ± 30	200 ± 35	229 ± 33	201 ± 20	ns
H_2S (U)[e]	10 ± 5.5	8 ± 3.7	10 ± 2.6	7 ± 3.6	6 ± 2.4	ns
Preference (%)	49.6 ± 12.2A	66.2 ± 1.1AB	69.6 ± 1.9B	67.2 ± 3AB	68.3 ± 2.1B	ns

[a] *P*-values obtained by ANOVA for the wines made with each yeast. (*** $P \leq 0.001$, * $P \leq 0.05$). *ns* not significant ($P > 0.05$)

[b] Data relative to the total *S. cerevisiae* like CFUs measured in a sample from tumultuous fermentation (2–3 days after the inoculation time)

[c] Data relative to the total microorganism CFUs measured 1 day after the inoculation time

[d] Data relative to the total microorganism CFUs measured 1 day after the inoculation time

[e] *U* arbitrary units. The data are the mean values and standard errors of 20 independent experiments (four uninoculated controls, four with JP88, four with E7AR1, four with SMR16-5A and four with RodM2H5-6D). Different letters (*A* and *B*) mean significantly different groups found with the Duncan test at $P < 0.05$

wild non-*Saccharomyces* yeasts and bacteria. They also increased the organoleptic quality and total acidity, and decreased the pH, volatile acidity and reducing sugars in both the red and the white wines. Therefore, technological improvement in wine-making can be obtained by using wine yeast strains produced in our low-cost plant.

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