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Autochthonous fermentation starters for the industrial production of Negroamaro wines

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Abstract The aim of the present study was to establish a new procedure for the oenological selection of *Saccharomyces cerevisiae* strains isolated from natural must fermentations of an important Italian grape cultivar, denoted as "Negroamaro". For this purpose, 108 *S. cerevisiae* strains were selected as they did not produce H_2S and then assayed by microfermentation tests. The adopted procedure made it possible to identify 10 strains that were low producers of acetic acid and hydrogen sulphide and showed that they completed sugar consumption during fermentation. These strains were characterized for their specific oenological and technological properties and, two of them, strains 6993 and 6920, are good candidates as industrial starter cultures. A novel protocol was set up for their biomass production and they were employed for industrial-scale fermentation in two industrial cellars. The two strains successfully dominated the fermentation process and contributed to increasing the wines' organoleptic quality. The proposed procedure could be very effective for selecting "company-specific" yeast strains, ideal for the production of typical regional wines. "Winery" starter cultures could be produced on request in a small plant just before or during the vintage season and distributed as a fresh liquid concentrate culture.

Keywords Wine microbiology · Yeast selection · *Saccharomyces cerevisiae* · Autochthonous yeast · Yeast biomass production

Introduction

The transformation of grape must into wine is due to a fermentation process naturally performed by native yeasts, which considerably contribute to the chemical and organoleptic properties of the wine [13]. The primary role of wine yeast is to promote the fast, entire and efficient transformation of grape sugar into ethanol, carbon dioxide and other important secondary metabolites, without the development of off-flavours. In the spontaneous fermentation of grape must there is a progressive growth pattern of non-*Saccharomyces* yeasts and a final stage in which the alcoholic fermentation process is completed by *Saccharomyces cerevisiae*, which becomes the dominant yeast species [1]. The diversity of native yeast strains can produce wines with different qualities and peculiar flavours [33]. However, to avoid the unpredictability of must spontaneous fermentation, the winemakers add active dried yeast culture to initiate and complete the fermentation process, thus impeding the development of non-*Saccharomyces* and favouring the full exhaustion of sugars and homogeneity in the final product.

Recently, there has been an increase in the use of autochthonous or local selected yeasts to control must fermentation. The employment of native, indigenous, selected local

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strains of *S. cerevisiae* as starters seems to be preferable [2, 22, 40], because these strains are adapted to all the conditions associated with a specific wine-production area [23] and are thus able to control the indigenous microflora during the fermentation process more efficiently.

Moreover, there is a growing demand to select, among the fermentative yeast flora, autochthonous yeast strains that ensure the maintenance and/or the improvement of the typical oenological and sensory characteristics which could be considered representative of an oenological region [29, 44, 56, 59].

In the present study, we propose a strategy to select *S. cerevisiae* strains from a large number of strains by key parameters indicative of their technological and qualitative characteristics. The *S. cerevisiae* population was isolated from the natural fermentations of Negroamaro musts, which derive from grapes sampled from the four most representative wine-producing areas in Salento (Apulia, Southern Italy). The yeast population was analysed and characterized by molecular assay and the identified strains were then subjected to physiological, oenological and technological tests after which of two indigenous *S. cerevisiae* strains, characterized by notable technological and oenological properties, were selected. The two selected strains were evaluated by laboratory tests and semi-industrial-scale fermentations to confirm whether they could be candidates as autochthonous fermentation starters. An optimized protocol was developed for the production of the two starter biomasses in order to test them in the industrial production of Negroamaro wine in two different wineries. To our knowledge, the present report is the first study of the *S. cerevisiae* population associated with Negroamaro grapes and of the employment of autochthonous starter cultures for the industrial production of a typical wine in Apulia.

Materials and methods

Natural fermentations

Negroamaro (*Vitis vinifera*) grapes (80 kg) were harvested in the Torchiarolo (Brindisi), Copertino (Lecce), Cutrofiano (Lecce) and Melissano (Lecce) areas, which are the most important areas for Negroamaro wine production in Apulia. Four spontaneous fermentations were carried out in 100-L sterile steel tanks in an experimental cellar with a temperature ranging between 24 and 26°C. Fermentations were monitored daily by measuring the Babo grade (°Ba; 1°Ba = 10 g/L of fermentable sugars in the must). Yeast populations were sampled at the end of alcoholic fermentation (0–1°Ba) and from the residual lees.

Yeast isolation

Yeast isolates were firstly screened for their ability to produce hydrogen sulphide. Tenfold dilutions from each must sample were plated onto Biggy agar (Sigma, USA) and then incubated at 28°C for 4 days. The isolates were screened according to the colony colour, thus making it possible to recognize distinct groups of strains: W (white colonies), BG (colonies of "beige" colour), LB (light brown colonies) and DB (dark brown colonies). For each of the four different must samples under analysis, at least 100 colonies were selected in categories W and BG. Isolates were combined with 20% sterile glycerol and then stored at −80°C for further characterization.

Identification of *S. cerevisiae* strains

Yeast total genomic DNA was prepared according to De Benedictis et al. [8]. The isolates belonging to the *Saccharomyces* genus (sensu stricto) were identified according to the length of the rDNA region spanning the 5.8S rRNA gene and flanking the internal transcribed spacers 1 and 2 [62]. Polymerase chain reaction (PCR) assays were carried out as previously described [3]. The amplified DNA products were visualized by agarose gel electrophoresis and then analysed with the Gel Compar 3.1 software (Applied Math, Kortrijk, Belgium). Thirty-six *S. cerevisiae* isolates from each sample were selected at random and characterized by interdelta typing [20]. Amplification products were separated by capillary electrophoresis and strain-specific amplicon profiles were identified as previously described [57]. Cluster analysis of the pairwise values was generated by the unweighted pair group method with arithmetic mean (UPGMA) algorithm, using the NTSYS software (Applied Biostatistics, USA). The described procedure was also adopted to determine the qualitative assessment of dominance of added yeast in experimental wine fermentations.

Microvinification assays

The identified *S. cerevisiae* strains were tested by microfermentation assays conducted in Negroamaro grape must. The must was filtered twice both through cheesecloth and a 0.22-μm membrane filter and, then, it was combined with 100 mg/L potassium metabisulphite. One litre of treated must was inoculated with 10^6 CFU/mL of yeast culture grown in the same must. Fermentations were carried out at 25°C and samples were weighed daily in order to follow the volatile CO_2 production until the weight was constant. An aliquot of fermented must (100 mL) was stored at −20°C, whereas the remaining wine was used for sensorial analysis. During fermentation,

hydrogen sulphide production was assayed according to the blackening of a PbAcO strip [27], thus making it possible to classify isolates as high (+++), medium (++), low (+) and no (−) sulphide producers. Fermentation rate (FR) and fermentation purity (FP) were calculated according to Ciani and Maccarelli [6]. Alcohol yield coefficient (AYC) was calculated as the amount of ethanol formed in relation to sugar consumed. Each fermentation experiment was carried out by performing three simultaneous and independent tests.

Enzymatic activities

Biogenic amines formation was determined as described by Nikolaou et al. [29]. Yeast strains were separately spotted on YPD agar plates, supplemented with 0.006% bromocresol purple and 1% of one chosen amino acid. The amino acids histidine, tyrosine, phenylalanine, tryptophan, lysine, leucine and arginine were tested. After incubation at 25°C for 7 days, amino acid decarboxylation appeared as a purple halo around the yeast colony. Screening for β-glucosidase activity was carried out on agar plates to which arbutin was added as a selective substrate [48]. Extracellular protease, polygalacturonase, pectinase, glucanase and xylanase production were determined as described by Strauss et al. [51].

Chemical and sensorial analysis

Wines and musts were analysed by Fourier transform infrared spectroscopy (FTIR), employing the WineScan Flex (FOSS Analytical, DK). Samples were centrifuged at 8,000 rpm for 10 min and then analysed following the supplier's instructions. The major volatile constituents [acetaldehyde, ethyl acetate, 2-methyl-1-propanol, higher alcohols (3-methyl- and 2-methyl-1-butanol), acetoin] were determined by gas chromatography, as previously described [8]. At the end of the alcoholic fermentation, wines were stored at 4°C for 10 days and, after discarding the deposited sediments, they were bottled. Bottles were stored for 25 days at 16–19°C and then a sample (50 mL) of each fermented must was poured into a tasting glass immediately before sensorial analysis. The panel was composed of seven judges, who evaluated the wines according to their flavour and colour profile. Each wine scored between 0 and 3 for each characteristic aroma: the aromas "wood", "fruity", "spicy", "mineral" were considered as positive, whereas "sulphide", "chemical", "herbal" and "animal" aromas were considered as negative [10]. Each sample scored between 1 and 4 for the red hue and between 0 and 3 for blue and brown shades [10].

Biomass production of starter cultures

Fed-batch experiments were carried out by employing the Biostat C fermenting system 30 L (Sartorius, Germany), using an optimized synthetic culture medium composed as follows: primary salts [$(NH_4)_2SO_4$ 6.0 g/L, $MgSO_4 \cdot 7H_2O$ 0.7 g/L, KH_2PO_4 4.0 g/L], yeast extract 0.5 g/L, secondary salts (1,000× = EDTA 22.5 g/L, $ZnSO_4 \cdot 7H_2O$ 6.8 g/L, $MnCl_2 \cdot 4H_2O$ 1.5 g/L, $CoCl_2 \cdot 6H_2O$ 0.5 g/L, $CuSO_4 \cdot 5H_2O$ 0.5 g/L, $Na_2MoO_2 \cdot 2H_2O$ 0.6 g/L, $CaCl_2 \cdot 2H_2O$ 7 g/L, $FeSO_4 \cdot 7H_2O$ 4.5 g/L, H_3BO_3 1.5 g/L, KI 0.2 g/L), vitamin cocktail (1,000× = α-biotin 0.1 g/L, D(+) calcium pantothenate 1.5 g/L, nicotinic acid 1.5 g/L, *myo*-inositol 38.0 g/L, thiamine hydrochloride 1.5 g/L, pyridoxal hydrochloride 1.5 g/L, *p*-aminobenzoic acid 0.30 g/L), sucrose 10 g/L. Biomasses were produced at 30°C, at constant pH (pH 5) and a dissolved oxygen concentration of 20%. The above conditions were constantly monitored and regulated on-line by a connected computer. Feeding strategy during fermentations was performed according to Porro et al. [34]. The biomasses produced were aseptically taken from the fermenter and kept at 4°C. The viability of the biomass stored at 4°C was determined at weekly intervals, by taking a sample under sterile conditions and making counts of viable cells by plating on YPD agar medium.

Industrial vinifications

To start must fermentations on a large scale, a 30-L initial yeast inoculum, corresponding to 1.5×10^{12} CFU/ml, was used. These cultures were transported to the winery and used as starters. Once in the winery, the yeast suspension was mixed with 300 kg of Negroamaro must and left for 6 h at room temperature. Then, the yeast-must mixture was added to 15 tons of Negroamaro must (sugars 226 g/L, 19.7°Ba, pH 3.35, assimilable nitrogen concentration 164.7 g/L). Industrial fermentations were carried out in 150,000-L stainless steel vessels. The alcoholic fermentation process was carried out at 25°C and its kinetics was monitored daily by measuring the concentration of reducing sugars. At the end of alcoholic fermentation (0°Ba), samples of wine and residual lees were collected for further analyses.

Statistical treatment of data

Significant differences among selected strains were determined for each chemical and volatile compound by analysis of variance (Tukey, $\alpha = 0.05$). The contribution of strains was estimated by principal component analysis (PCA). Statistical data processing was performed using the XLSTAT 6.0 statistical package.

Results and discussion

The purpose of the present study was to analyse the genetic and oenological characterization of *S. cerevisiae* strains associated with the natural fermentation of Negroamaro must and to select suitable autochthonous starter cultures for the improvement of oenological production of typical regional wines. Grape samples were collected in the four most representative Negroamaro-producing areas in Apulia. Natural fermentations were carried out on an experimental scale (100 kg of must) and the sampling of microflora was performed at the end of the alcoholic fermentation process (<1°Ba). The first step of the selection procedure concerned the selection of the isolates unable to produce hydrogen sulphide by applying a phenotypic assay on selective solid medium [64]. More than 900 yeast colonies collected at the end of each natural fermentation studied were analysed, allowing the isolation of no or low H_2S producers (W and BG phenotypes on Biggy agar): 100 isolates from Copertino, 138 isolates from Cutrofiano, 104 isolates from Melissano, 108 isolates from Torchiarolo.

The second step of the optimized protocol consisted of the identification at the genus level of the above four isolate populations, obtained by PCR analysis of their rDNA region. The molecular analysis of yeast isolates' rDNA made it possible to confirm that all the 450 colonies isolated at the end of fermentations belonged to the genus *Saccharomyces*, which is in full agreement with data obtained from previous studies in other Mediterranean areas [11, 19, 30, 44].

In the third step of the selection procedure, 36 isolates were randomly chosen from each of the four low-H_2S-producer groups, as this sample size was a statistically significant sample of the biomass present in each of the fermentation phases [28, 35, 36, 58]. The PCR-based assay, i.e. the amplification of interdelta regions, allowed the identification of 108 different strains: 27 different strains in the population of Copertino, 29 distinct strains in the population of Cutrofiano, 24 different strains in the population of Melissano, 28 distinct strains in the population of Torchiarolo (Fig. 1). These strains were deposited in the Institute of Sciences of Food Production(ISPA) Collection (http://www.ispa.cnr.it/Collection/). The four analysed populations were denoted by a high intraspecific polymorphism on the yeast population present at the end of fermentation that was comparable to those described in other investigations of *S. cerevisiae* population dynamics during must fermentation [15, 37, 44, 55]. Moreover, in each sample we noticed the absence of a dominant strain at the end of fermentation, this evidence being in agreement with the results of a similar study, in which a high number of different strains of *S. cerevisiae* were reported in spontaneous fermentation [49].

In the fourth step of the proposed selection protocol, all the identified strains were tested in microfermentation assays in order to evaluate strain-specific technological and oenological properties [4, 24, 29]. We suggest a strategy for the evaluation of data obtained from the microfermentation assay, which consists of the primary cross-evaluation of three major descriptors and, then, a sequential further selection which is based on the evaluation of several other secondary descriptors. Therefore, the following oenological and technological parameters were primarily considered and their values were employed as discriminative for the selection of autochthonous yeast strains for their use in controlled fermentations: acetic acid production (<0.6 g/L), because the formation of this volatile acid produced by *S. cerevisiae*, generally in high concentrations, is definitely undesirable [12]; total sugar consumption, because the presence of residual sugar in the must (>4 g/L) is indicative of an incomplete fermentation [33]; lack of H_2S production during fermentation, perceptible on a sensory level with a strong and unpleasant odour of rotten eggs, also called reduced odour [64].

The primary screening indicated that the indigenous yeast strains 6911, 6920, 6925, 6931, 6952, 6953, 6975, 6978, 6979 and 6993 satisfied the above requested parameters. These strains were selected to be further characterized. Their main oenological and technological properties and the results of quantitative analysis of the main chemical compounds present in musts fermented by each one of them are shown in Tables 1 and 2, respectively.

Several studies [9, 17, 47, 54] have emphasized the existence of a differential contribution among selected strains of *S. cerevisiae* in determining the flavour and taste of wines, and that yeasts make an important contribution during fermentation to the expression of the character of the grape variety. Indeed, the microfermentation tests made it possible to evaluate, at the same time, the main technological properties that affect the progress of the fermentation [45] and also oenological and aromatic properties that influence the quality of wines [52]. The study of fermentation kinetics was based on the evaluation of the initial and final rate of fermentation [45]. The initial rate of fermentation is a symptom of an ability for rapid colonization at the beginning of fermentation [65]. The ten selected strains demonstrated a high initial fermentation rate (values between 0.8 and 2.2 g) when compared with data obtained in previous studies [60].

After fermentation, taking into account the whole duration of fermentation, the consumption of sugars was high and comparable to the commercial reference strain (Table 2). Hydrogen sulphide is the end product of sulphate reduction and the initial product of the biosynthesis of sulphur amino acids [39]. Yeasts use only part of the H_2S to conduct this biosynthesis, so that the excess is excreted in
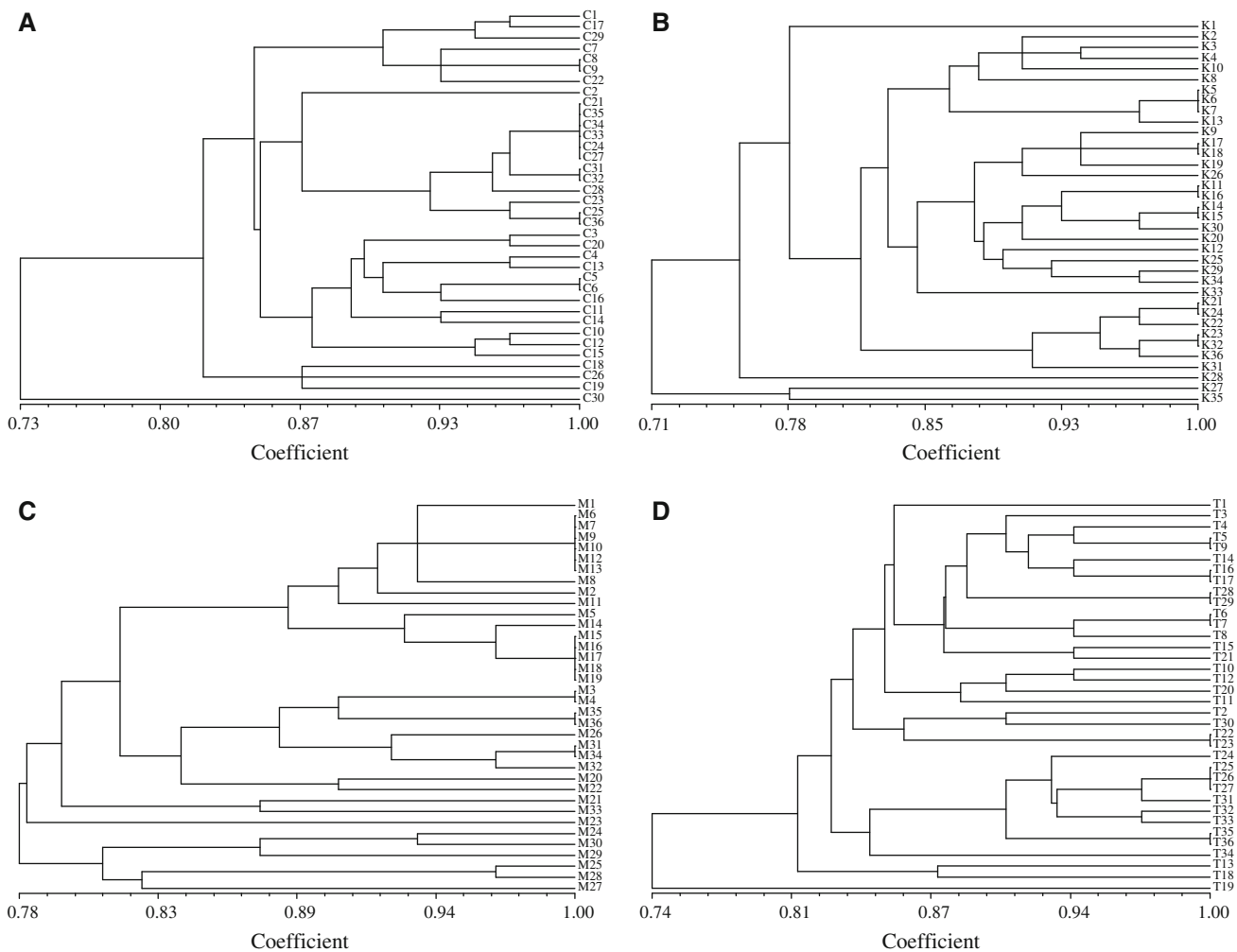
Fig. 1 UPGMA dendrograms generated by cluster analysis of interdelta region patterns obtained from the *S. cerevisiae* strains isolated during the later stages of spontaneous fermentation of Negroamaro grapes from **a** Copertino, **b** Cutrofiano, **c** Melissano, **d** Torchiarolo. Calculated percentages of homology are given under each dendrogram

the must. Consequently, during fermentation, all strains of *S. cerevisiae* produce amounts of this compound, for which the threshold of acceptability is 80 µg/L. Several factors, such as the chemical composition of the must and fermentation conditions, influence the production of hydrogen sulphide in the wine, which is still a strain-dependent property [45]. Indeed, the ten selected strains were preselected for their low production of H_2S by a plate assay and they further confirmed this property during experimental must fermentation (Table 1).

Other parameters evaluated were the fermentation purity, the alcohol yield and the fermentation rate (Table 1), which are desired strain-specific properties for an industrial fermentation starter [60], because high concentrations of ethanol are one of the causes of stuck fermentations. High tolerance to alcohol allows the yeast strain to drive the fermentation correctly avoiding stuck fermentation [50] and production of undesired secondary compounds [16]. The

glycerol formed by yeast during fermentation is one of the main components of wine, in which it is usually found in concentrations ranging from 5 to 8 g/L [32]. The glycerol has a key role in the formation of the bouquet of wine, as it improves the balance and structure of wine [31]. The ten selected strains were found to produce satisfactory amounts of glycerol, with values of up to 8.39 g/L (Table 2).

In order to quantify the presence of higher alcohols produced by fermentation in must, the concentrations of 2-methyl-1-propanol (isobutyl alcohol) and combined amyl alcohols (2-methyl-1-butanol and 3-methyl-1-butanol) were evaluated (Table 3). The latter ranged from 31.30 mg/L (strain 6978) to 84.07 mg/L (strain 6925), indicating that all strains could positively contribute to the aromatic complexity of wine [43]. Another very important constituent of the major polar compounds is ethyl acetate. The values found for this ester ranged between 3.25 mg/L for 6979 and 7.75 mg/L for 6920 (Table 3). Hence, at these concentra-

Table 1 Main oenological and technological properties determined in one commercial (CM) and ten autochthonous *S. cerevisiae* strains

Isolate	ITEM nr.	FP	AYC	FR	H₂Sᵃ	Foamᵃ
10C	6911	0.05	0.57	0.8	−	−
19C	6920	0.02	0.60	0.9	−	−
2K	6925	0.03	0.56	1.9	−	−
14K	6931	0.03	0.56	1.8	−	−
2T	6952	0.02	0.57	2.2	−	−
3T	6953	0.04	0.58	1.6	−	−
3M	6975	0.04	0.58	1.2	−	−
10M	6978	0.04	0.57	1.3	−	−
11M	6979	0.04	0.58	1.5	−	−
1TZ	6993	0.02	0.59	2.1	−	−
CM		0.04	0.58	0.8	+	−

Data, measured at the end of fermentation, represent the average of three replicates

ITEM ISPA's Agro-Food Toxigenic Fungi Culture Collection, *FP* fermentation purity [volatile acidity (g/L)/ethanol (% v/v)], *AYC* alcohol yield coefficient [alcohol (% v/v/initial sugars (%) − final sugars (%)], *FR* fermentation rate (g of CO_2/day, after 3 days of fermentation)

ᵃ H₂S and foam production: absent (−); low (+), high (++), very high (+++)

tions, the contribution of this ester to the aroma of wines could be considered positive [38, 53]. Acetaldehyde was the most important carbonyl compound produced during fermentation and its concentrations ranged from 10.02 mg/L (strain 6920) to 19.71 mg/L (strain 6925). The amount of acetoin, produced by the tested strains, ranged from 3.63 mg/L for 6931 to 6.68 mg/L for 6911 (Table 3) and these concentrations were consistent with those described in a similar study by Romano and Suzzi [46].

The fermented musts were also subjected to sensory analysis (Fig. 2). In all samples, a *fruity* aroma was identi-fied with a maximum score for strain 6920, as well as the *spicy* descriptor, with the exception of 6978 and 6979 strains. The negative aromatic descriptors *sulphide*, *chemical* and *herbal* were identified only in samples 6952, 6953 and 6979. The visual examination (not shown) revealed a red tinge in all samples, with scores ranging from a minimum of 2 (6975) to a maximum of 4 (6920). Four samples (6952, 6993, 6925, 6978) exhibited shades of blue, whereas only the samples 6953, 6931, 6979 and 6975 showed shades of brown.

In the sixth step of the selection procedure, the ability to produce enzymes of oenological interest was evaluated in the selected strains by performing different physiological tests, thus enabling identification of strains able to produce useful extracellular enzymes during wine fermentation (Table 4). None of the ten selected strains decarboxylated several amino acids, thus indicating that they were not able to produce biogenic amines. The characterization of yeast strains for the production of biogenic amines has strong importance for the careful selection of starter cultures that do not cause any potential risk to consumer health [5]. All the selected strains, with the exception of the 6931, showed lichenase activity. This activity has high technological relevance, because it can favour wine filterability as a greater proportion of the grape glucan contains 1,3–1,4 linkages [51]. The strains 6952 and 6993 demonstrated that they could produce 1,3-*β*-D-glucanases. The 6920 strain was able to produce the enzyme specifically degrading pectin, which may cause problems in the wine industry by giving rise to turbidity and viscosity during the vinification process [61].

PCA was applied to the data matrix comprising concentrations of nine compounds (2-methyl-1-propanol, acetic acid, acetoin, ethyl acetate, fructose, acetaldehyde, isoamyl

Table 2 Concentration of major chemical compounds in wines obtained with ten autochthonous and one commercial (CM) strain of *S. cerevisiae*

Strain	Malic (g/L)	Acetic (g/L)	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Ethanol (g/L)
Must	5.44 ± 0.03	ND	106.68 ± 0.01	106.53 ± 0.01	0.91 ± 0.08	ND
6911	4.90 ± 0.07	0.55 ± 0.00	ND	0.35 ± 0.01	5.24 ± 0.05	97.77 ± 0.11
6920	5.10 ± 0.00	0.22 ± 0.00	ND	0.19 ± 0.05	8.39 ± 0.05	101.23 ± 0.09
6925	4.83 ± 0.00	0.33 ± 0.00	ND	0.39 ± 0.09	7.61 ± 0.00	96.61 ± 0.10
6931	4.65 ± 0.00	0.41 ± 0.00	ND	0.42 ± 0.07	6.63 ± 0.00	96.11 ± 0.00
6952	4.56 ± 0.14	0.30 ± 0.17	ND	0.38 ± 0.03	8.36 ± 0.41	97.21 ± 1.08
6953	4.70 ± 0.14	0.47 ± 0.17	ND	0.34 ± 0.03	8.31 ± 0.41	99.10 ± 1.08
6975	4.95 ± 0.08	0.46 ± 0.15	ND	0.37 ± 0.08	6.10 ± 0.01	98.62 ± 0.06
6978	4.89 ± 0.07	0.53 ± 0.00	ND	0.29 ± 0.06	5.34 ± 0.05	97.81 ± 0.11
6979	4.24 ± 0.00	0.52 ± 0.03	ND	0.40 ± 0.08	7.59 ± 0.06	99.52 ± 0.14
6993	4.05 ± 0.14	0.26 ± 0.17	ND	0.20 ± 0.04	8.38 ± 0.41	100.96 ± 1.08
CM	4.90 ± 0.07	0.47 ± 0.01	ND	0.30 ± 0.08	5.14 ± 0.05	98.71 ± 0.04

Values are the mean of three injections of each replicate (*n* = 9); the standard deviation values (±) are indicated

ND not detectable

Table 3 Concentration of major volatile compounds in wines obtained with ten autochthonous and one commercial (CM) strain of *S. cerevisiae*

Compound	Strain											
	Must	6911	6920	6925	6931	6952	6953	6975	6978	6979	6993	CM
Acetaldehyde	0.98 ± 0.37	15.40 ± 0.33	10.02 ± 1.12	19.71 ± 0.59	10.53 ± 0.30	15.77 ± 0.44	16.48 ± 0.26	11.37 ± 0.39	11.67 ± 0.33	10.57 ± 0.23	10.11 ± 0.17	14.02 ± 0.06
Ethyl acetate	0.93 ± 0.75	3.57 ± 0.20	7.75 ± 0.33	4.13 ± 0.23	3.99 ± 0.09	4.39 ± 0.37	4.01 ± 0.47	3.32 ± 0.18	3.33 ± 0.08	3.25 ± 0.11	7.26 ± 0.37	7.62 ± 2.17
2-Methyl-1-propanol	0.09 ± 0.13	10.44 ± 0.10	8.68 ± 0.31	11.31 ± 0.05	9.75 ± 0.14	11.01 ± 0.04	11.54 ± 0.16	13.17 ± 0.23	33.55 ± 0.23	13.77 ± 0.33	10.95 ± 0.22	17.78 ± 0.14
Amyl alcohols	0.24 ± 0.27	78.14 ± 0.52	72.84 ± 1.51	84.07 ± 0.67	71.01 ± 0.83	82.28 ± 0.56	83.33 ± 0.26	73.33 ± 0.25	31.30 ± 0.27	71.68 ± 0.21	77.09 ± 0.18	77.06 ± 0.19
Acetoin	1.25 ± 1.37	6.68 ± 0.36	3.89 ± 2.10	5.19 ± 0.65	3.63 ± 0.31	5.13 ± 0.36	5.57 ± 0.19	6.90 ± 0.38	5.90 ± 0.28	4.50 ± 0.24	3.68 ± 0.34	5.46 ± 0.82

Values expressed in mg/L are the mean of three injections of each replicate ($n = 9$); the standard deviation values (±) are indicated

alcohols, glycerol and ethanol) for the ten identified strains (Fig. 3). Data concerning the hydrogen sulphide production (Table 2) and sensory analysis (Fig. 2) were not included in the PCA, because they were not continuous. Along the first component, the samples were clearly grouped in three clusters. Samples 6978 and 6911 were grouped in a single cluster on the right part of the plot, whereas samples 6920 and 6993 were grouped into a more scattered cluster on the other side of the plot. The others samples (6979, 6975, 6931, 6953, 6952, 6925) were closely grouped in the central part on PC1. The loadings of each compound on the principal components show clearly that ethanol and glycerol are mainly responsible for the cluster of 6920 and 6993, whereas the isoamyl alcohols and acetaldehyde characterize the samples 6952, 6925 and 6953 and the acetic acid and 2-methyl-1-propanol are responsible for the differentiation of the other strains 6979 and 6975.

The result of PCA confirmed the evidence given by the analytical assays, making it possible to conclude that, among all the indigenous strains of *S. cerevisiae* analysed, the strains 6993 (native to Torchiarolo) and 6920 (native to Copertino) retained the technological, chemical and aromatic properties required for their possible use as an industrial starter for Negroamaro wine production. The 6920 and 6993 strains were, therefore, used for the inoculation of large-scale fermentation in two local industrial cellars.

Yeast biomasses were produced by performing a fed-batch fermentation in a 30-L bioreactor. Process optimization during fed-batch cultivation was based on the following parameters: pH, temperature, aeration rate and the feed profiles of sugar. Many different carbohydrate feed stocks can be used for yeast production, such as cane or beet molasses, which are low-cost by-products from sucrose production companies. However, molasses are dense and viscous liquids, with a composition highly variable depending on the sucrose refining procedure and on the year's weather conditions [18], which can affect the yield and quality of the yeast biomasses produced. In this study, a novel culture medium has been set up to ensure optimal growth conditions, using commercial sucrose as the carbon source and a minimal medium added to salts and vitamins. The fed-batch culture prevents inhibition problems due to toxic products of cell metabolism and the accumulation of genetic mutations, reducing also the possibility of contamination [25]. The principle adopted to establish the optimal duration of the two phases of fermentation ("batch" and "feed") was based on evidence, already documented in the literature [21], that changes in fermentative metabolism during fermentation correspond to changes in the pH of the medium culture. The shift in the pH regime, from acidic to alkaline, during the first phase of fermentation (batch), made it possible to identify the moment when reducing sugars were about to be totally catabolised, thus indicating the

Fig. 2 Sensory analysis of must fermented with the selected strains of *S. cerevisiae*. "Positive" descriptors are represented on the *upper side* of the graph whereas "negative" descriptors are represented on the *lower* negative scale. The description of *bars*, corresponding to each aromatic descriptor, is reported in the panel
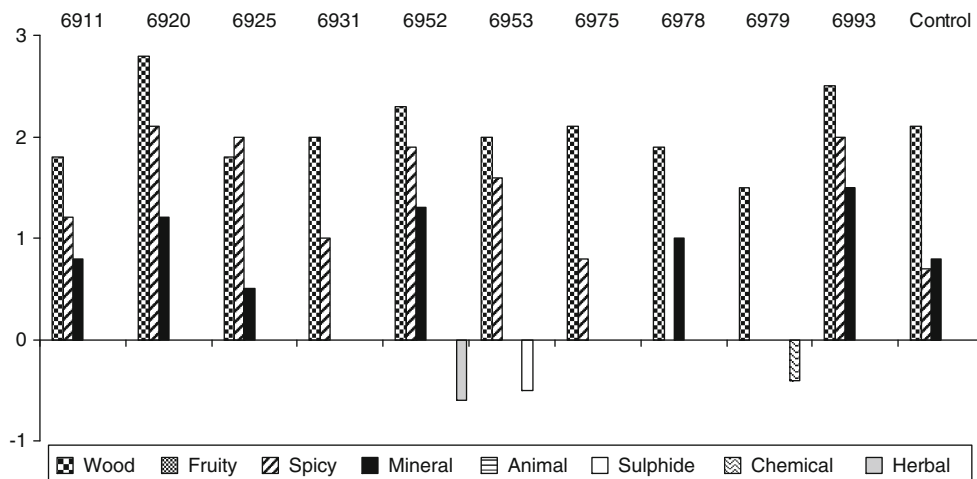

Table 4 β-Glucosidase, decarboxylase, protease, pectinase, glucanase and xylanase activity associated with the selected strains and determined by specific plate assay

Strains	β-Glucosidase	AA decarboxylation						Protease	Pectinase	Glucanase		Xylanase
		His	Tyr	Phe	Trp	Leu	Arg			Pachyman	Lichenan	
6911	−	−	−	−	−	−	−	−	−	−	+	−
6920	−	−	−	−	−	−	−	−	+	−	+	−
6925	−	−	−	−	−	−	−	−	−	−	+	−
6931	−	−	−	−	−	−	−	−	−	−	−	−
6952	−	−	−	−	−	−	−	−	−	+	+	−
6953	−	−	−	−	−	−	−	−	−	−	+	−
6975	−	−	−	−	−	−	−	−	−	−	+	−
6978	−	−	−	−	−	−	−	−	−	−	+	−
6979	−	−	−	−	−	−	−	−	−	−	+	−
6993	−	−	−	−	−	−	−	−	−	+	+	−

+ presence; − absence

need to begin to supply the above carbon source to the fermenting culture (second stage or feed phase).

The optimized protocol made it possible to obtain biomasses ranging from 2.4×10^{13} to 4.5×10^{13} cells (final volume 30 L), a sufficient amount to inoculate 16–30 tons of grape must. Levels of viability of yeast biomass were relatively stable during the time considered, showing only a minimum loss of vitality (17%), even after 35 days' storage at 4°C (Fig. 4). Indeed, microscopic analysis revealed that the biomasses produced were composed of yeasts with homogeneous cell size and morphology, whereas contaminations by acetic bacteria, coliforms, enterobacteria or filamentous fungi were never observed (data not shown). The use of a fresh yeast (liquid) as the inoculum, instead of dehydrated active dry wine yeast (ADWY) commonly used in the food industry, made it possible to improve the fermentation process. Indeed, fresh yeasts in liquid form are ready to use, in contrast to ADWY, which requires a laborious and delicate process of rehydration in water [42].

However, liquid cultures require a longer lead time, as they have to be grown on demand.

These large-scale experiments were conducted in two winery cellars of Salento, each of which provided about 150 hL of grape must. The data corresponding to the fermentation performance of the two isolates used and their ability to dominate the fermentation indicated that these two autochthonous yeast strains possess the fundamental properties required for starter cultures [42]. In fact, the fermentations progressed regularly and sugar depletion was completed in 6 days (data not shown). The dominance of inoculated strains was confirmed by the analysis of the interdelta region polymorphism. Data show that strains 6920 and 6993 were able to dominate the yeasts naturally present in the must, with a high predominance (70%) in total yeast population at the end of fermentation (Fig. 5).

The results of chemical and gas chromatographic analyses of the two wines produced are shown in Table 5. Both strains produced low concentrations of acetic acid (volatile

Fig. 3 PCA performed employ-
ing as variables the data obtained
by the chemical analysis of must
fermented with the selected
strains. *Arrows* indicate the posi-
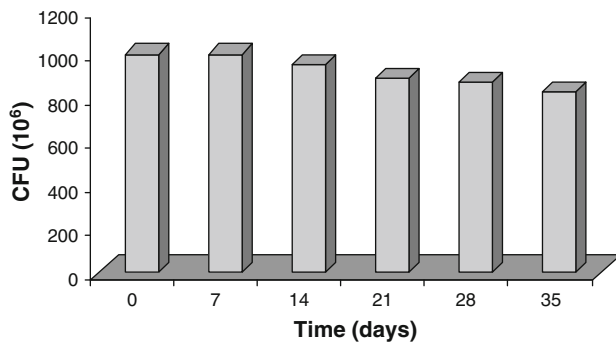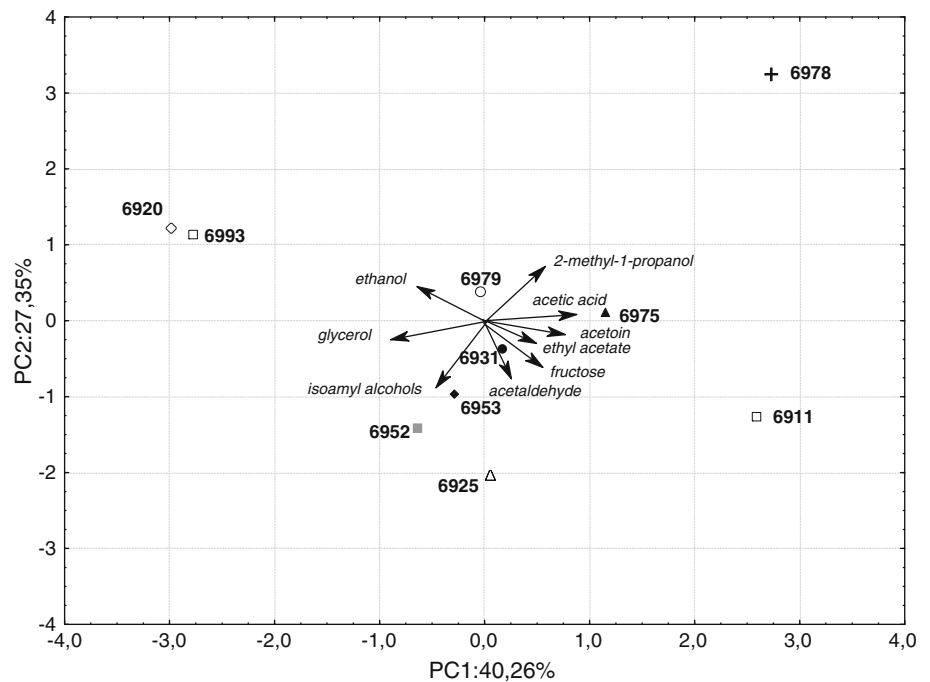tion of the different analysed
variables

Fig. 4 Viability of biomass stored at 4°C determined at weekly inter-
vals

acidity) with values of 0.27 ± 0.00 g/L for 6993 strain and
0.28 ± 0.01 g/L for 6920 strain. The total acidity was
higher in must fermented by strains 6993 (6.36 g/L); in fact
in this sample the pH values were lower (3.6). In both sam-
ples, tartaric acid was the most abundant organic acid
detected (Table 5). Both strains were able to metabolize
sugars at a final concentration below the value of 2.5 g/L.
Furthermore, the two strains showed a high alcohol power
[actual alcoholic content (g/100 mL)], with values of
13.64% (strain 6993) and 13.49% (strain 6920).

The colour of a wine can be considered as the sum of the
three colour components: yellow (420 nm), red (520 nm)
and blue (620 nm) [14]. For the wines made in the two vini-
fications, the colour intensity fluctuated between 2.83 for
strain 6993 and 2.01 for strain 6920, although the percent-
age of red was maximal for strain 6920 (54.27%) and low-
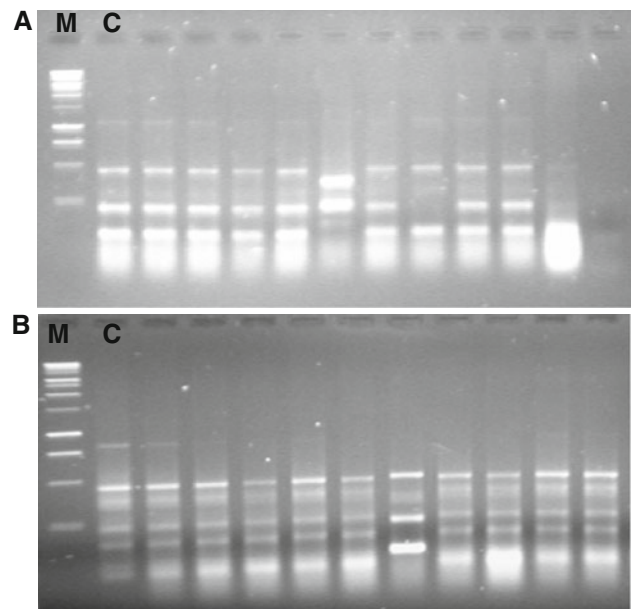est for strain 6993 (51.93%). It is clear that in the latter

Fig. 5 Electrophoretic profiles of inter-δ region patterns obtained
from ten *S. cerevisiae* strains randomly isolated at the end of the two
large-scale fermentations inoculated with strain 6920 (**a**) and 6993 (**b**).
The strain-specific profile for the 6920 and the 6993 strains is reported
in each panel (*C*). *M* molecular weigh marker (1 kb DNA Ladder;
Promega, USA)

sample the contributions due to minority colour compo-
nents of yellow (32.76%) and blue (15.32%) were also sig-
nificant.

The concentrations of the main volatile components of
wine were determined by gas chromatographic analysis
(Table 5). The values of acetaldehyde ranged from a

Table 5 Main parameters characterizing the chemical properties and concentration of major volatile compounds in wines obtained by the autochthonous 6920 and 6993 *S. cerevisiae* strains

	6993	6920
Alcohol (g/100 mL)	13.64 ± 0.00	13.49 ± 0.00
Reducing sugars ((g/L)	2.27 ± 0.03	2.25 ± 0.04
Total acidity (g/L)	6.36 ± 0.02	5.88 ± 0.01
Volatile acidity (g/L)	0.27 ± 0.00	0.28 ± 0.01
Glycerol (g/L)	9.66 ± 0.05	9.32 ± 0.00
Methanol (ml/100 mL)	0.32 ± 0.00	0.33 ± 0.00
Malic (g/L)	3.15 ± 0.16	3.22 ± 0.22
Lactic (g/L)	1.31 ± 0.04	1.23 ± 0.03
Tartaric (g/L)	3.18 ± 0.08	3.37 ± 0.07
Citric (g/L)	0.23 ± 0.01	0.17 ± 0.01
pH	3.63 ± 0.00	3.72 ± 0.00
Total polyphenols (mg/L)	3,804 ± 31	3,675 ± 23
Anthocyanins (mg/L)	156.00 ± 3.00	232.00 ± 6.00
Absorbance at 420 nm	0.926 ± 0.01	0.670 ± 0.00
Absorbance at 520 nm	1.468 ± 0.01	1.093 ± 0.00
Absorbance at 620 nm	0.433 ± 0.01	0.251 ± 0.01
Acetaldehyde (mg/L)	1.87 ± 0.2	10.44 ± 0.2
Ethyl acetate (mg/L)	47.61 ± 2.2	54.92 ± 2.2
2-methyl-1-propanol (mg/L)	18.82 ± 0.0	15.03 ± 0.3
Pentanol (combined) (mg/L)	95.74 ± 0.1	187.24 ± 0.2
Acetoin (mg/L)	10.36 ± 0.3	8.66 ± 0.3

Values are the mean of three injections; the standard deviation values (±) are indicated

1.87 mg/L (strain 6993) to 10.44 mg/L (strain 6920). Ethyl acetate was detected in quantities in the range of 47.61 mg/L (strain 6993) and 54.92 mg/L (strain 6920), it being higher than that detected during microvinification assays probably because its biosynthesis was enhanced by the anaerobic conditions of large-scale fermentation [42].

As regards the amyl alcohol products, the amounts detected ranged from 95.74 mg/L (6993) to 187.24 mg/L (strain 6920). The concentrations of acetoin ranged between 8.66 mg/L (strain 6920) and 10.36 mg/L (strain 6993).

Conclusions

A novel protocol for the selection of *S. cerevisiae* starter cultures and for the preparation of their biomasses has been optimized and validated by industrial-scale production of Negroamaro wine in two different local wineries. The procedure consisted of the following steps: (1) selection of no-H$_2$S-producer isolates; (2) yeast identification at species level; (3) yeast typing at strain level; (4) strain evaluation by fermentation test; (5) chemical analysis of fermented

must; (6) estimation of strain-specific enzymatic properties; (7) statistical analysis of obtained data; (8) starter biomass production and its use as inoculum for industrial-scale must fermentations. The selected strains were always able to dominate the fermentation process and to produce a final product characterized by excellent oenological and organoleptic features.

The selected starter cultures could hereafter be applied to better investigate the use of industrial starters composed of a consortium of either *S. cerevisiae* [63] or *Saccharomyces* and non-*Saccharomyces* mixed strains [7, 8]. These approaches are nowadays considered as possible strategies to enhance the organoleptic complexity of wines, which is naturally produced by the complex microbial process operated by cultivable and non-cultivable microbial species during the winemaking process [41].

In conclusion, the proposed selection procedure can be very effective for the preparation of "company-specific" yeast starter cultures, which could represent the ideal solution to enhance the specific features of typical regional wines. "Winery" starter cultures could be produced on demand in low-costs plants [26], just before the vintage season, and distributed to the wineries as a fresh liquid concentrate culture.

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