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Autochthonous Fermentation Starters for the Production of Aglianico Wines

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Aglianico cultivar is among the most ancient black grape varieties native of Campania, a region of the Southern of Italy. Traditionally wines are produced by natural fermentation carried out by the autochthonous non-*Saccharomyces* and *Saccharomyces* yeasts present on the grapes and in cellar environment respectively. The aim of this work is the selection and the exploitation of new combinations of microorganisms, isolated from the native microflora of Aglianico grapes and musts, to improve the organoleptic and sensory characteristics of the produced wine. The yeasts isolated and identified by morphological, biochemical and molecular methods mainly belonged to the genera *Saccharomyces, Kloeckera, Candida, Metschnikowia, Hanseniaspora* and *Rhodotorula*. Among them two indigenous strains of *Saccharomyces cerevisiae* and *Metschnikowia fructicula* were characterized through laboratory tests and semi-industrial-scale fermentations, and selected as good candidates for autochthonous fermentation starters. The fermentations carried out with the new starters showed some positive differences compared to those carried out with commercial yeasts: in fact the selected strains efficiently completed the fermentations and positively affected the wine quality. In particular, the indigenous yeasts increased the must total acidity, reaching the expected values of pH and alcohol content, without producing excessive levels of acetic acid. They also enhanced the colour and the content of polyphenols, flavonoids and anthocyanins. HPLC analysis showed a significant increase of gallic acid, catechin and resveratrol concentrations. The results demonstrated that the two strains successfully dominated the fermentation process and contributed to improve the wines' organoleptic quality preserving the peculiarities of these typical regional wines.

1. Introduction

Aglianico cultivar is a red grape variety of Greek origin autochthonous of Irpinia, a geographical area of Southern Italy that has an established wine industry. The oldest and simplest way for wine production is the spontaneous fermentation that is carried out by the indigenous yeasts present on the surface of the grape and on the cellar equipments. (Clemente-Jimenez et al., 2004; Fleet, 2008). The grape epiphytic microflora is responsible for the early stages of the must fermentation and is mainly composed by non-*Saccharomyces* yeasts with a low fermentative power. As the fermentation proceeds, the fermentative *Saccharomyces* yeasts, become predominant, replacing the non-*Saccharomyces* ones. The *Saccharomyces* "sensu stricto" strains are "winery" yeasts present on the winemaking equipments. Each winery is characterized by its own fermentative microflora originated by the selection occurred among the species "resident" in the cellar. Recently, to plan and standardize the winemaking process, the utilization of microbial starter composed by selected *S. cerevisiae* yeasts

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have been suggested. However, this procedure inhibits the growth of the non-*Saccharomyces* strains that greatly contribute to the wine quality (Jolly et al., 2003). In fact, they are involved in the determination of properties such as the colour and aromatic complexity of the wine that are important determinants of product typicality. Several researchers promoted the utilization of such selected yeasts strains in order to get mixed fermentation inocula (Ciani et al., 2010) and many studies revealed significant positive differences in the qualitative and quantitative volatile compounds composition of the wines obtained with a guided fermentations compared to those produced with spontaneous yeasts (Calabretti et al. 2011; Comitini et al. 2011). The aim of our work was the selection and the exploitation of new combinations of autochthonous *Saccharomyces* and non-*Saccharomyces* yeast strains, isolated from the native microflora of Irpinian Aglianico grapes and musts, in order to produce an Aglianico wine with improved organoleptic and sensory characteristics, thus preserving the peculiarities of this typical regional wine.

2. Materials and Methods

2.1 Chemicals

The culture media for the isolation, the reactivation and the growth of different yeast strains were purchased from Oxoid (Hampshire, UK). Chemical reagents and solvents are all of analytical grade and were from SIGMA-Aldrich (St.Louis, MO, USA).

2.2 Isolation and molecular identification of yeasts

To isolate the different yeasts populations, serial dilutions of Aglianico grape samples, previously homogenized in sterile Ringer solution, were plated on three different solid agar media: YPD, WL-nutrient and Lysine-agar On WL medium, yeast species have been distinguished by different colony morphologies and colours. The isolation of non-*Saccharomyces* species has been carried out on Lysine medium, in which *Saccharomyces spp* could not grow (Calabretti et al., 2011). The yeast species identification was performed by the analysis of the D1/D2 domain of 26S rDNA sequence. The genetic region was PCR amplified directly from individual yeast colony following the protocol, as described by Arroyo-Lopez et al. (2006). The standard primers utilized were the commonly referred as NL1 and NL4 in the literature (O'Donnell, 1993).

2.3 Determination of the yeast fermentative power

One hundred mL of pasteurized must (100 °C for 30 min) was inoculated with a 1 % (v/v) microbial biomass suspension (24 h growth). The must fermentation tests were carried out in shake-flasks incubated at 28 °C. The flasks weight decrease was measured every day until a constant weight has been reached and maintained for three days.

2.4 Yeasts selection for the resistance to sulphur dioxide

The tests were carried out on Aglianico must, it was pasteurized for 30 min and inoculated with a 1 % of microbial biomass suspension (24 h growth). Potassium metabisulfite (MBK) was used as the source of sulphur dioxide and the yeasts resistance at concentrations of 100 and 250 mg/L was tested. The tests were performed at 28 °C for 7 days (Costanti et al. 1998).

2.5 Growth of yeast strains

The selected yeast strains were inoculated in 100 mL YPD liquid medium. The growth was carried out at 28 °C in shake-flasks at 200 rev min^{-1} (rpm) in an orbital shaker (New Brunswick Scientific Co., Inc., USA) for 24 h. The obtained yeast suspension was used to inoculate, at (1 % v/v) 10 L of YPD liquid medium in a pilot plant fermenter. The obtained biomass was stored at 4 °C.

2.6 Winemaking process

Aglianico grapes were harvested at 18.4 °Brix, destemmed, pressed and then fermented with their skins. MBK was added at a final concentration of 100 mg/L to the juice and, after the addition of 20 mg/L of Trenolin® Rouge DF pectolitic enzyme (Erbslöh Geisenheim AG, Geisenheim, Germany), it was warmed at 18 °C for 18 h. The must was then divided in five 30 L tanks for fermentation heated up to 26 °C. All the tanks with the exception of one that was utilized as control were inoculated with different combination of the selected autochthonous strains *Saccharomyces cerevisiae* AGYP37 and

Metchnikowia fructicola AGYP28*,* with or without the commercial yeast (C.Y.), (about 3 x 10^6 CFU/mL). The control was inoculated with 0.15 g/L (3.2 x10^{10} CFU/g) of dry *S. cerevisiae* Oenoferm® Structure (Erbslöh) previously rehydrated according to manufacturer instructions. At the beginning of the fermentation a mobilisator and a nutrient (0.2 g/L Vita*Drive*®, Vitamon® Combi from Erbslöh) were added. Moreover, in the middle of fermentation, another aliquot of the nutrient, at the same concentration, was added. The fermentation process was completed in 10 days and when the residual sugars concentration reached a value smaller than 2 g/L, 60 mg/L of MBK was added. The wine was decanted with aeration for 3 days followed by 10 days without aeration. Sugars evaluation was carried out by densitometric analysis using a Babo mustimeter.

2.7 Analysis of total polyphenols, flavonoids and anthocianins

Total polyphenol content was determined using the Folin-Ciocalteu reagent (Waterman and Mole, 1994) following a micro scale as described by Arnous et al. (2002). The absorbance at 750 nm was recorded, and the total polyphenol concentration was calculated from a calibration curve using gallic acid as standard and expressed as mg gallic acid equivalents/L (GAME). The total flavonoid content was determined by the aluminium chloride colorimetric method as reported by Marinova et al. (2005). The assay was carried out utilizing aliquots of 1 mL of Aglianico wine diluted with methanol (ratio 1:9) and catechin solutions in 80 % methanol as standards. The absorbance at 510 nm was measured and the total flavonoid concentration was calculated from a calibration curve using catechin as standard and expressed as mg catechin equivalents/L (CE). Total and colored anthocyanins measurements were performed using a well-established spectrophotometric method (Arnous et al. 2002).

2.8 HPLC analysis of phenolic compounds

Phenolic compounds analysis was performed by HPLC/UV with a C18 column Hypersil (Thermo Electron Corporation, Bellefonte, PA, North America). The injected sample volume was 20 µL. Peak identifications were confirmed from retention times and direct comparison to pure standards. The solvent flow rate was 0.9 mL/min and the mobile phase was a four-step linear solvent gradient system (0–5 min, 10 % B; 5-40 min, 45 % B; 40–45 min, 100 % B; 45-50 min, 100 % B; 50-55 min, 10 % B) using 2 % acetic acid in water as solvent A and 0.5 % acetic acid in 50 % acetonitrile as solvent B. UV detection was carried out at 280 nm and 254 nm.

3. Results and Discussion

Microbiological analysis of Aglianico must microflora revealed the presence of species mainly belonging to the *Hanseniaspora, Kloeckera, Metchnikowia, Candida* and *Saccharomyces* genera according to Beltran et al. (2002). In particular *Hanseniaspora, Kloeckera, Metchnikowia* were predominant in the must before the addition of MBK, after the inoculation and in the first days of the fermentative process. These species, except *Metschnikowia* spp.*,* together with *Saccharomyces* spp. were present during the early stages of the alcoholic fermentation, due to their ability to tolerate alcoholic concentrations up to 3 or 4 % alcohol by volume (Hierro et al., 2006). A non-*Saccharomyces* (AGYP28) and a *Saccharomyces* (AGYP37) strain were selected for their resistance to sulphur dioxide and the alcohologenic and fermentative power (data not shown). The experimental winemaking processes carried out with the Aglianico must inoculated with the starters obtained from a combination of the isolated yeasts and the commercial *S. cerevisiae* (C.Y.) showed that the autochtonous yeasts were able to trigger and complete the alcoholic fermentation leaving a sugar residue smaller than 2 g/L (Figure 1).

Moreover content in polyphenols, flavonoids and anthocyanins was increased compared to that obtained with C.Y., as reported in Figure 2 and 3. In particular, the best yield in polyphenols and flavonoids were obtained using a combinations of the two autochthonous yeasts (AGPY37, AGYP28) (Figure 2).

It is well known that polyphenol compounds have positive effects against the reactive oxygen species (ROS), that are involved in many diseases, such as cardiovascular and chronic kidney pathologies, certain types of cancer, neurological disorders, inflammation and hypertensive conditions (Zenebe et al., 2001).

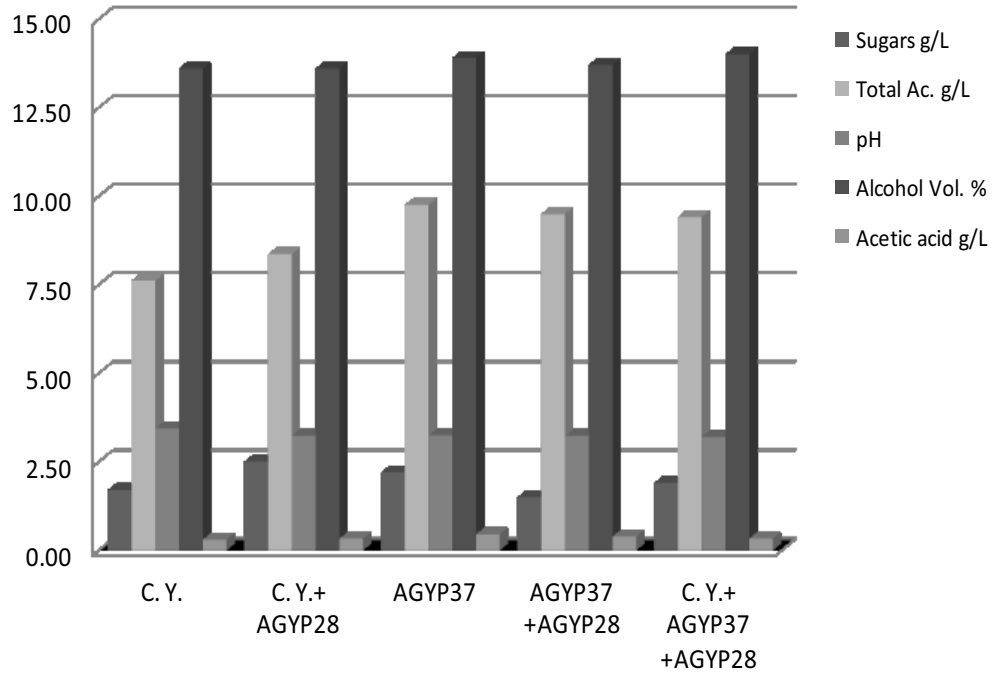

Figure 1: Oenological parameters in Aglianico fermentations

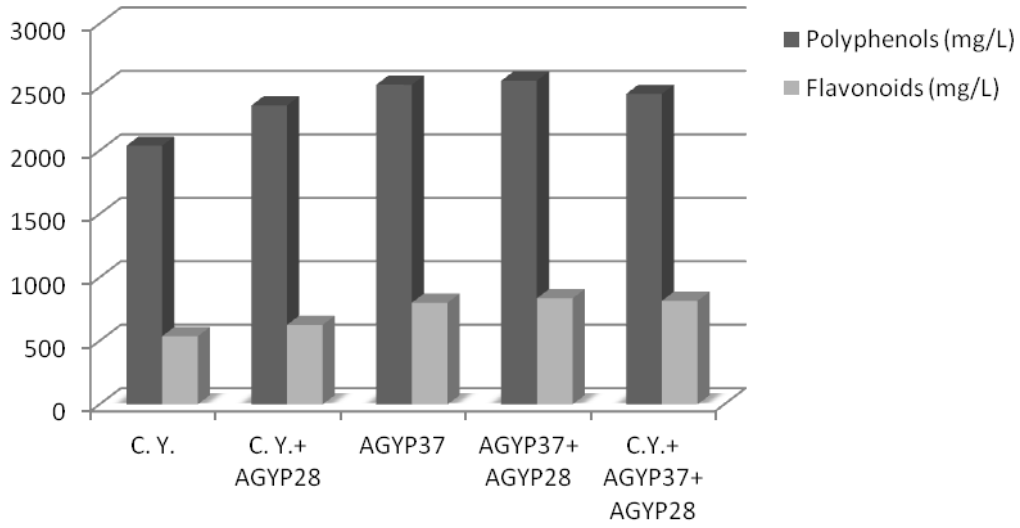

Figure 2: Concentrations of polyphenols and flavonoids in Aglianico fermentations

Therefore, the production of wine with an enhanced content of these molecules could be desirable. The flavonoid content generally increases utilizing the autochthonous yeasts reaching the highest value for the fermentation with *S. cerevisiae* AGPY37 and *M. fructicola* AGYP28. On the other hand, the maximum concentration of total antocyanins was reached with a the combination of C.Y. and AGYP28 (Figure 3).

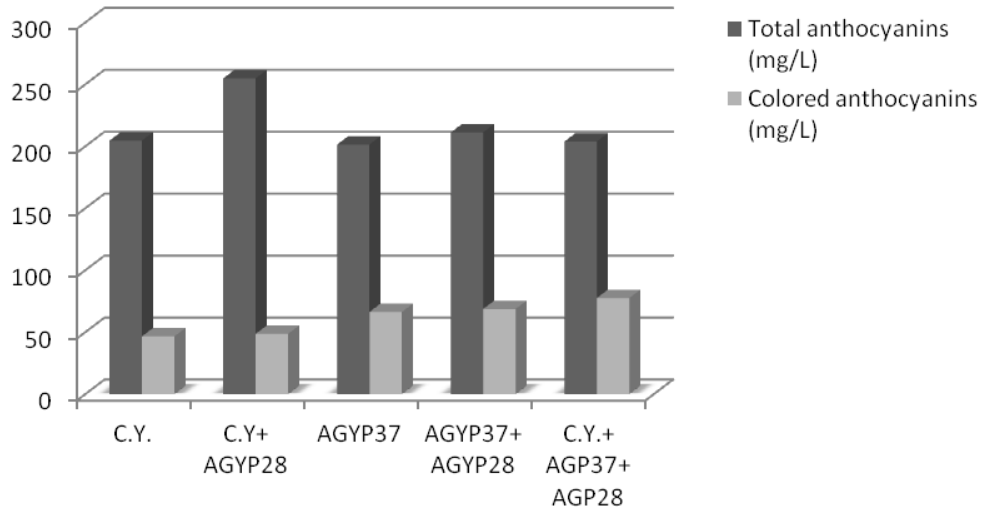

Figure 3: Concentrations of anthocyanins in Aglianico fermentations

The results of HPLC analysis obtained for the Aglianico wine are reported in Table 1. The fermentation experiments with native yeasts showed that the gallic acid content was higher in wines obtained with one or both autochthonous strains. The presence of a larger amount of gallic acid could be a consequence of hydrolysis of gallotannins and esters of gallic acid with glucose. In the fermentations carried out with indigenous strains, the level of catechins increased of about 25 % while the epicatechin content remained unchanged. In the wine produced with the combination of all three yeasts, the level of catechins did not change while the epicatechin content drastically decreased. Coumaric acid and cinnamic acid concentrations always substantially decreased in the presence of native yeasts. The resveratrol content was higher in wines produced with the three strains or with a combination containing the native yeast AGPY37. On the contrary, wine obtained with the combination C.Y./AGYP28 had the lowest content of resveratrol.

Table 1: Final concentrations of phenolic compounds in Aglianico wines obtained after the different fermentations

Compounds mg/L	C.Y.	C.Y. AGPY28	AGPY37	AGPY37 AGPY28	C.Y. AGPY37 AGPY2
Gallic Acid	46.1 ± 2.0	54.2 ± 2.1	61.3 ± 4.2	59.2 ± 3.2	51.1 ± 3.0
Catechin	75.3 ± 4.4	94.2 ± 5.2	97.1 ± 6.1	95.3 ± 5.1	72.2 ± 5.2
Chlorogenic acid	n.d.	n.d.	36.3 ± 2.4	n.d.	n.d.
Epicatechin	57.3 ± 3,4	68.4 ± 4.3	60.2 ± 4.1	57.1 ± 4.2	5.1 ± 0.3
Coumaric acid	18.0 ± 1.0	6.1 ± 0.4	14 ± 0.9	11 ± 0.9	9 ± 0.6
Resveratrol	6.0 ± 0.5	4.3 ± 0.4	7.0 ± 0.5	8.1 ± 0.5	8.4 ± 0.1
Cinnamic acid	11.0 ± 1.1	3.1 ± 0.1	3.0 ± 0.1	7.3 ± 0.5	0.5 ± 0.1

Interestingly, the wine produced with AGYP37 had the highest level of gallic acid and catechin and only in this wine chlorogenic acid was detected. As the absence of this compound, usually present in wine, may be correlated with the presence of an enzymatic activity capable of splitting the ester-linkages between cinnamic and quinic acid, it could be suggested that this activity is not repressed in the fermentation with AGYP37. Finally, the combination of the two native strains led to production of wines with the highest resveratrol level, also in the presence of C.Y.

In conclusion, the results demonstrated that the two strains successfully dominated the fermentation process and contributed to improve the wines' organoleptic quality preserving the peculiarities of these typical regional wines.

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