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Indigenous *Saccharomyces cerevisiae* yeasts as a source of biodiversity for the selection of starters for specific fermentations

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Abstract. The long-time studies on wine yeasts have determined a wide diffusion of inoculated fermentations by commercial starters, mainly of *Saccharomyces*. Although the use of starter cultures has improved the reproducibility of wine quality, the main drawback to this practice is the lack of the typical traits of wines produced by spontaneous fermentation. These findings have stimulated wine-researchers and wine-makers towards the selection of autochthonous strains as starter cultures. The objective of this study was to investigate the biodiversity of 167 *S. cerevisiae* yeasts, isolated from spontaneous fermentation of grapes. The genetic variability of isolates was evaluated by PCR amplification of inter-δ region with primer pair $\delta 2/\delta 12$. The same isolates were investigated for characteristics of oenological interest, such as resistance to sulphur dioxide, ethanol and copper and hydrogen sulphide production. On the basis of technological and molecular results, 20 strains were chosen and tested into inoculated fermentations at laboratory scale. The experimental wines were analyzed for the content of some by-products correlated to wine aroma, such as higher alcohols, acetaldehyde, ethyl acetate and acetic acid. One selected strain was used as starter culture to perform fermentation at cellar level. The selection program followed during this research project represents an optimal combination between two different trends in modern winemaking: the use of *S. cerevisiae* as starter cultures and the starter culture selection for specific fermentations.

1. Introduction

The conversion of grape must into wine is promoted by a fermentation process naturally carried out by indigenous yeasts [1]. Until about the 1980s, the contribution of yeasts to wine production was seen as a relatively simplistic concept. The main function of wine yeasts is to guarantee the rapid and complete conversion of grape sugar into ethanol, carbon dioxide, and many secondary metabolites, avoiding the production of off-flavours. Spontaneous fermentation is characterized by the activity of different yeast species/strains, even if almost invariably it is dominated by strains of the yeast *Saccharomyces cerevisiae*. It is well known that the diversity of native yeast strains is responsible for the production of wines with different qualities and peculiar flavours. Yeast species and, within each species, different strains exhibit wide differences in volatile compound production, accounting for the differences in composition and in taste of wine. Although this yeast diversity can contribute to the wine complexity and can produce unique-flavoured wines [2, 3], the dynamics of a spontaneous fermentation is often unpredictable and some non-*Saccharomyces* species can also produce undesirable compounds. Therefore, in the wine-making industry the growth of undesirable yeasts is controlled by addition of sulphur dioxide to musts and inoculation with selected strains of *Saccharomyces,* mainly *S*. *cerevisiae*.

The *S. cerevisiae* strains, involved in fermentation, play an important role in the characteristics of wine [4,5]. Although many flavour components derive directly from the grapes, the essential part of a wine flavour is produced during the alcoholic fermentation. In fact, the composition and the sensory quality of the resulting wine are due to the diversity of *S. cerevisiae* strains. Different strains of *S. cerevisiae* can produce significantly different flavour profiles when fermenting the same must [6] and this is a consequence of the differential ability of wine yeast strains to release varietal volatile compounds from grape precursors and the strain-specific capability to *de novo* synthesise yeast-derived volatile compounds [7].

Today, most wine is produced using selected commercial strains of *Saccharomyces*, but many wine-researchers and winemakers prefer the use of selected autochthonous strains of *S. cerevisiae* as starters. In fact, it seems that the use of commercial dried yeasts reduced the biodiversity of strains performing natural fermentation, and, as a consequence of this, a reduction of the resulting wine complexity. Actually, also small wineries are interested in the selection of yeasts from their own environment for use as starter cultures. Extensive ecological surveys using molecular methods have been carried out with the aim of selecting new yeasts better adapted to local fermentation conditions [8]. The indigenous *S. cerevisiae* strains are better acclimated to micro-area conditions of the wine production region and therefore they can more easily

dominate on the natural biota. Furthermore, the use of locally selected yeast strains with strain-specific metabolic characteristics could positively affect the final quality of wine [9,10] and ensure the maintenance of the typical sensory properties of wines deriving from a specific area.

The selection protocol of indigenous strains, which have to be used as starter cultures for specific fermentations, includes the study of different parameters. Among the desirable and traditional oenological criteria, the following are the most frequently evaluated during the selection protocol: high fermentation performance, resistance to, and low production of, sulphur dioxide; low production of hydrogen sulphide and low volatile acidity; resistance to ethanol; positive influence on wine aroma. In recent studies addressed to the selection of indigenous starter cultures, another trait considered was the evaluation of strain ability to dominate natural yeast population (strain implantation), always present during must fermentation [11,12]. "Matera DOC" is a little wine-production of Basilicata, Southern Italy, which awarded its Registered Designation of Origin (DOC) classification in 2005. The types of wines produced are six, three red and three white. Some producers carry out spontaneous fermentation of grape musts, whereas other cellars utilize commercial yeasts strains as starters to reduce the risk of wine spoilage and uniform wine quality.

The aim of this study was to investigate the biodiversity and the oenological properties of *S. cerevisiae* isolated during spontaneous fermentation of grapes collected in this specific area in order to select those strains with specific characteristics and well adapted to the cellar environment to be used as starters in winemaking. The selection program followed during this research project represents an optimal combination between two different trends in modern winemaking: the use of *S. cerevisiae* starter cultures which can produce wine characterized by a reproducible quality and the selection of the starter culture for specific fermentations in function of the vine variety characteristics.

2. Material and methods

2.1. Yeasts

One hundred and sixty-seven *Saccharomyces cerevisiae* isolates were studied. The yeasts were previously isolated from spontaneously fermented grapes (Aglianico variety), collected from vineyard grown under organic farming methods, cultivated near Pollino National Park (Basilicata Region, Southern Italy). The isolates were maintained on YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 2% (w/v) agar).

2.2. Technological characterization

The isolates were tested for their tolerance to different antimicrobial compounds, such as ethanol (EtOH), sulphur dioxide (SO_2) and copper sulphate ($CuSO_4$). These tests were performed as described by Mauriello et al. [6]. Hydrogen sulphide (H_2S) production was evaluated by inoculating the yeasts on bismuth-containing indicator medium BIGGY agar (Oxoid) and the plates were incubated at 26 °C for 2 days. On this medium the production level of H_2S is related to browning of yeast colonies: H_2S-positive strains exhibit brown or black colonies, while H_2S-negative colonies are white.

All the data obtained by technological characterization were converted into non-dimensional values, assigning for each isolate the following values:

- 0 for parameters exhibited at low level,
- 1 at middle level,
- 2 at high level. The values were submitted to the cluster analysis, using the Paired Group method with Euclidean distance. The statistical package used was PAST software ver. 1.90 [13].

2.3. Genotypic characterization

The genetic variability among the 167 isolates was evaluated by amplification of inter-δ region with primer pair δ2-δ12 [14,15], following the protocol described by Capece et al. [10].

2.4. Fermentation trials at laboratory-scale with selected *S. cerevisiae* strains

Twenty selected *S. cerevisiae* isolates were tested in inoculated fermentations at laboratory scale in comparison to *S. cerevisiae* commercial strain used in the cellars. The fermentations were performed in 130-ml Erlenmeyer flasks filled with 100 ml of grape must, added with 50 mg l^{-1} of SO_2. Each strain was inoculated in grape must at a concentration of 10^6 cells ml-1, from a pre-culture grown for 48 h in the same must. The fermentation was performed at 26 °C and the fermentative course was monitored by measuring weight loss, determined by carbon dioxide evolution during the process. At the end of the process (stable weight), the wine samples were refrigerated at 4 °C to clarify the wine, racked and stored at −20 °C until required for analysis. All the experiments were performed in duplicate.

During this experiment, the qualitative amount of H_2S formed during fermentation was determined by evaluating the browning degree of commercial lead acetate test strips, inserted in the top of fermentation flasks. The degree of blackening of the strips correlates to the amount of H_2S produced during fermentation [16].

The content of some secondary compounds influencing wine aroma were analyzed by direct injection gas chromatography of 1 μl of experimental wines, by following the method described in Capece *et al.* [12]. Levels of these compounds were quantified by internal standardization (calibration curves) using Agilent ChemStation Software. Levels of secondary compounds determined in the experimental wines were submitted to statistical analysis by descriptive Box plots and whiskers, using PAST software ver. 1.90. The experimental wines were analyzed for the total and free SO_2 content, which was measured iodo-metrically by the Ripper procedure [17].

2.5. Inoculated fermentations at pilot scale

Fermentations at pilot scale were carried out in a cellar producing "DOC Matera wine" during the 2013 vintage.

One indigenous strain (selected during this study) and the commercial strain commonly used in the cellar were tested. The fermentations were performed in sulphite (50 mg l^{-1}) Aglianico grape must (240 g l^{-1} sugar, pH 3.5,), by inoculating 10^6 cells ml^{-1} in 100^{-1}-vats and the fermentation processes were monitored daily by determining sugar and temperature. The implantation ability of inoculated strains during the fermentative process was tested by following the protocol described by Capece et al. [10]. Conventional chemical parameters of wines, such as total acidity, volatile acidity, glucose and fructose, alcohol content, lactic and malic acids, were measured using Fourier Transfer Infrared WineScan (FOSS, Hillerød, Denmark), whereas the content of secondary compounds affecting wine aroma was detected as previously reported.

3. Results

3.1. Technological characterization

In the first step of this research, 167 *S. cerevisiae* yeasts, isolated from spontaneous fermentation of Aglianico grapes, were investigated for characteristics of oenological interest, such as resistance to chemical compounds potentially present during the process (SO_2, EtOH, $CuSO_4$) and H_2S production. All the data related to the technological characterization were statistically elaborated, obtaining the dendrogram reported in Fig. 1. As shown in the figure, the strains were distributed in 14 groups, some of which were composed by one/two isolates (1, 3, 5, 7, 12, 13 and 14). The most numerous groups were 4, 7 and 9, which grouped 31, 30 and 43 isolates, respectively (Table 1). Generally, the isolates exhibited a high tolerance to SO_2 (Table 1); in fact, the majority tolerated the highest dose tested (300 mg l^{-1}), whereas only 3 isolates were inhibited by concentrations higher than 100 mg l^{-1} (groups 1 and 7, Fig. 1). On the contrary, the main percentage of isolates was characterized by low EtOH tolerance (growth in presence of 12% v/v) (all the groups reported in Fig. 1, except 8, 9 and 13) and only few isolates (belonging to group 10) tolerated the highest dose tested (18% v/v). A certain variability was found for copper resistance; about 50% of isolates were inhibited by lowest doses tested (0–100 mmol l^{-1}) and the tolerance levels of other isolates were distributed among the other concentrations tested. As regards the H_2S production, generally the isolates were characterized by medium production level as, after growth on BIGGY agar, they developed colonies characterized by brown color. Only the strain M1-47 (group 5, Fig. 1) was no-H_2S producer; in fact, this strain developed white colonies on BIGGY agar.

3.2. Genotypic characterization

The evaluation of genetic polymorphisms among 167 isolates was carried out by PCR analysis of inter-δregion by using the primer pair $\delta 2/\delta 12$. Six different interdelta-profiles were found among the 167 yeasts; the profiles obtained are reported in Fig. 2, in which a different letter was assigned to each pattern that differed from the others in at least one intense band.

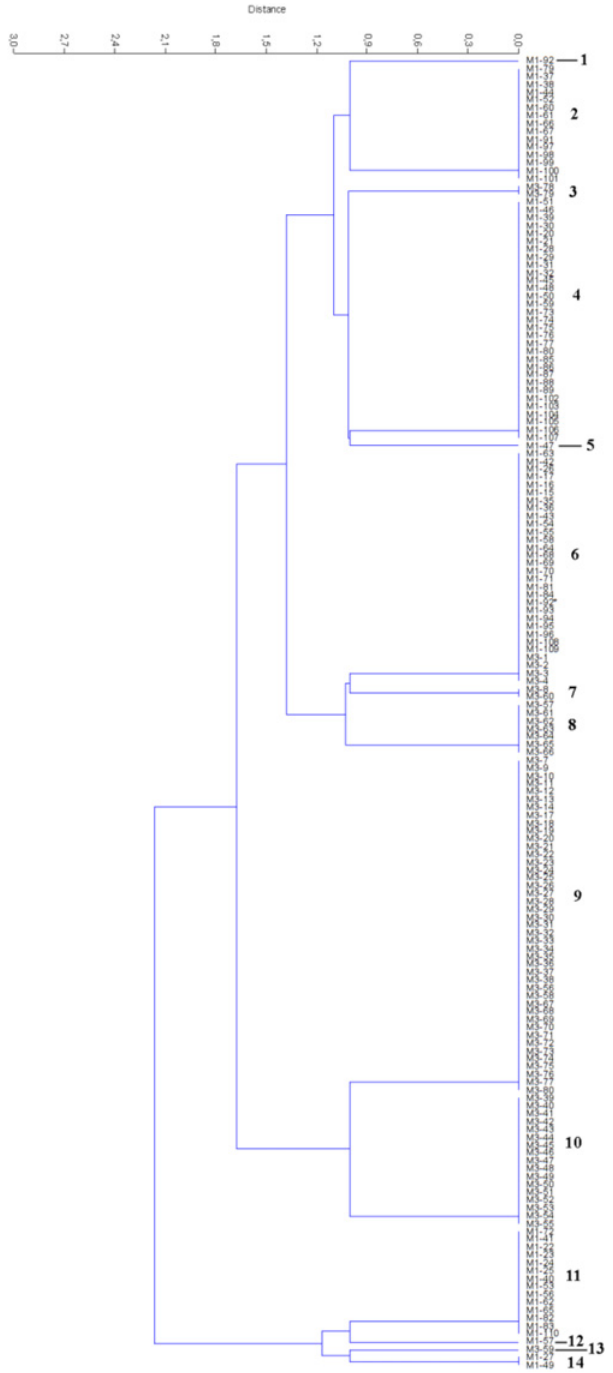


Figure 1. Dendrogram obtained after a hierarchical agglomerative cluster analysis performed on data of technological characterization of 167 *S. cerevisiae* isolates.

The profile "a" was the most common (exhibited by 59% of analyzed isolates), followed by pattern "b" (29% of yeasts), whereas other profiles were exhibited by few strains. The profiles "d" and "e" were specific of single strains. The comparison of results obtained with technological and genetic characterization revealed that isolates grouped in the same technological group showed the same molecular profile (see Table 1), although isolates sharing the same molecular profile were subdivided in different technological groups. For example, isolates exhibiting the inter-δ profile "a" were distributed in 6

Table 1. Characteristics of 167 *Saccharomyces cerevisiae* isolates, grouped according to technological characterization.

Group[a]	Resistance			H_2S[b]	N° of Isolates	Inter-δ profile
	EtOH*	SO_2**	Cu***			
1	12	100	200	M	1	a
2	12	200	200	M	15	b
3	12	300	100	M	2	a1
4	12	300	200	M	31	b
5	12	300	200	L	1	b
6	12	200	< 100	M	30	a
7	12	100	100	M	2	a
8	14	200	0–100	M	7	a
9	14–16	300	100	M	43	a
10	18	300	100	M	17	a
11	12	300	400–500	H	14	c
12	12	300	200	H	1	d
13	14	300	400	M	1	e
14	12	300	400	M	2	b

a = Technological groups, reported in Fig. 1; b = production evaluated on BIGGY agar; M = medium; L = low; H = high; * (% v/v); ** (mg/L); *** (μmol/L of $CuSO_4$).

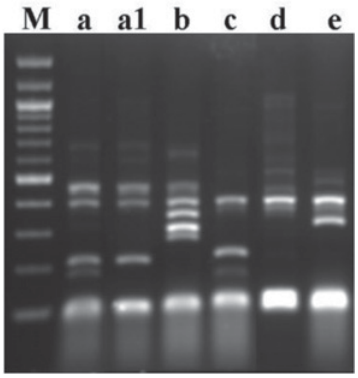
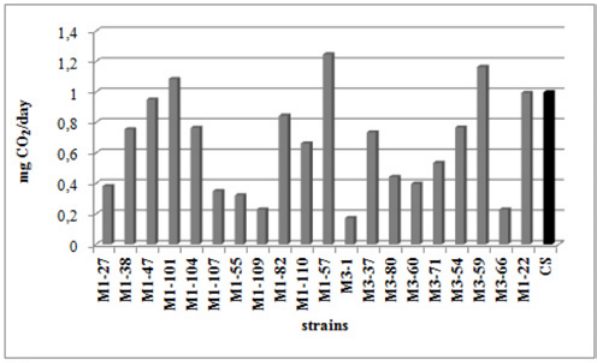


Figure 2. Molecular profiles obtained by amplification of inter-δ region with primer pair $\delta 2/\delta 12$ of indigenous *S. cerevisiae* isolates. M: 100 bp marker (Biolabs).

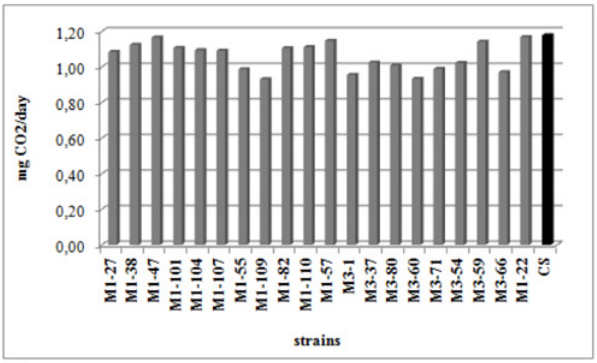
technological groups (1, 6, 7, 8, 9 and 10, Table 1). Probably, the analysis of the inter-δ region by using only a primer pair was not enough to discriminate the isolates, as previously reported [10, 18]. At this purpose, the use of other typing molecular techniques, which analyze different regions of yeast genetic patrimony, will be necessary to correctly evaluate the genetic variability among the 167 *S. cerevisiae* isolates.

3.2.1. Fermentation trials at laboratory-scale with selected S. cerevisiae strains

On the basis of the technological characteristics, twenty *S. cerevisiae* isolates, representative of the different clusters reported in Fig. 1, were selected and subjected to further characterization. In order to analyze the fermentative performance of selected strains, the *S. cerevisiae* isolates were tested in inoculated fermentations at laboratory scale in comparison to a commercial starter widely used in the wineries (CS). The fermentations were performed in the same grape must from which the yeasts were isolated; the process course was monitored by evaluating carbon dioxide evolution and the process was completed after 12–14 days. The Figs. 3a and b reports for each strain the fermentative rate, expressed as amount of



(a)



(b)

Figure 3. Fermentation rate expressed as g CO_2/day, measured at the first 2 days (a) and at the end (b) of the fermentation.

CO_2 produced for each fermentation day, measured at the first two days of fermentation (Fig. 3a) and at the end of the process (Fig. 3b). High variability in fermentation rate was recorded during the first step of the process. Some strains (M1-109, M3-1 and M3-66) produced very low level of CO_2/day, whereas other strains (M1-101, M1-57 and M3-59) produced more than 1 g CO_2/day and these values were higher than the fermentation rate exhibited by the commercial starter. When the fermentation rate was measured at the end of the process (Fig. 3b), the differences among the strains were reduced and indigenous

Table 2. Characteristics of experimental wines obtained by inoculating 20 indigenous *S. cerevisiae* strains in comparison to commercial starter (CS). ADE = acetaldehyde; N-PR= *n*-propanol; ACET = acetic acid; ISM = isoamyl alcohol.

Strains	Sulphur compounds*			Aromatic compounds (mg l^{-1})			
	SO_2(T)	SO_2 (F)	H_2S	ADE	N-PR	ACET	ISM
M1-22	12	12	2	21,38	29,86	570,28	241,15
M1-27	12,8	9,6	2	72,54	33,07	206,52	185,38
M1-38	10,4	9,6	3	48,74	27,15	143,51	189,66
M1-47	55,2	9,6	0	136,41	85,65	217,48	153,02
M1-55	15,2	10,4	0	44,46	44,37	180,00	190,65
M1-57	12	12	1	23,31	33,91	299,01	199,12
M1-82	12	12	1	24,27	29,32	414,81	238,72
M1-101	12	11,2	4	82,05	25,42	165,18	163,95
M1-104	10,4	9,6	4	89,77	34,64	228,89	169,06
M1-107	9,6	9,6	4	60,68	28,88	235,51	176,05
M1-109	12,8	11,2	1	31,33	32,94	243,23	194,54
M1-110	11,2	11,2	1	44,15	32,05	373,02	243,64
M3-1	14,4	10,4	2	36,15	36,40	224,58	179,92
M3-37	16,8	10,4	1	43,79	40,67	166,16	179,03
M3-54	14,4	12,8	3	42,44	43,04	241,89	180,27
M3-59	14,4	12	2	38,47	34,03	227,72	208,91
M3-60	12	10,4	0	21,79	46,35	239,40	146,22
M3-66	14,4	12,8	1	30,25	38,30	210,42	198,99
M3-71	13,6	11,2	2	52,67	42,92	210,25	170,76
M3-80	16,8	12	2	47,12	42,68	232,23	191,46
CS	13,6	12	3	39,61	34,31	359,89	207,66

* Concentration of total (T) and free SO_2 (F) was expressed as mg l^{-1}; H_2S production was expressed as arbitrary values in function of blackening degree of lead acetate strips.

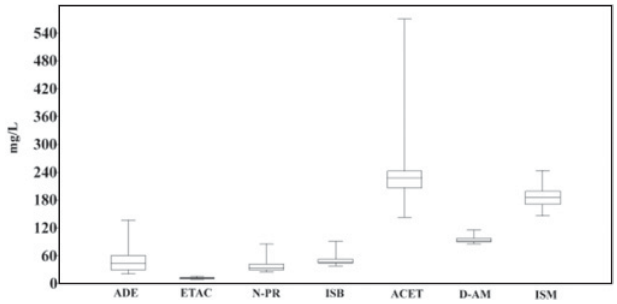


Figure 4. Box plot representing the variability of secondary compounds determined in the experimental wines produced by inoculating 20 *S. cerevisiae* strains. ADE = acetaldehyde; ETAC = ethyl acetate; N-PR = *n*-propanol; ISB = isobutanol; ACET = acetic acid; D-AM = D-amyl alcohol; ISM = isoamyl alcohol.

strains exhibiting fermentation rate comparable to those of commercial starter were found (i.e. M1-22, M3-59, M1-57 and M1-47). The lowest value was exhibited by the same strains showing the lowest fermentation rate at the first two days of the process.

The experimental wines obtained were analyzed for the content of some by-products correlated to the organoleptic quality of wine. The metabolites determined by gas-chromatographic analysis were: higher alcohols (*n*-propanol, isobutanol, amyl alcohols), acetaldehyde, ethyl acetate and acetic acid. The determination of metabolite levels showed considerable differences in the obtained wines (Fig. 4); in particular, high variability in the levels of acetic acid, isoamyl alcohol and acetaldehyde was found. Although the variability found in the production level of acetic acid and isoamyl alcohol, in all the obtained wines the level of these compounds was comprised in the desirable range. In fact, the maximum amount of acetic acid detected in the experimental wines was 570 mg l^{-1}, below the level considered negative (> 600 mg l^{-1}) and the maximum amount of isoamyl alcohol was 240 mg l^{-1} and this compound exert a negative influence on wine aroma when the level exceeds 300 mg l^{-1}. As regards the acetaldehyde content, only the wine produced by inoculating the strain M1-47 contained a very high amount of this compound (135 mg l^{-1}).

As regards the SO_2 content of wine (Table 2), the maximum amount detected was 16.8 mg l^{-1} of total SO_2; in the majority of wines, the SO_2 was present in free form.

A different behaviour was found for the strain M1-47; in fact, the wine produced by inoculating this strain contained the highest level of SO_2 (55.2 mg l^{-1}); furthermore, in this wine the majority of SO_2 is present in bound form as the level of free SO_2 is very low (9,6 mg l^{-1}). This might be due to the high production level of acetaldehyde (Table 2), which represents one of the principal sulphite-binding compounds. Furthermore, this strain is characterized by non-production of hydrogen sulphide (Table 1, group 5) and the highest production level of *n*-propanol.These results confirmed the data previously obtained [16], reporting the close relationship between high production of *n*-propanol and strain incapacity to produce hydrogen sulphide.

The results regarding the evaluation during fermentation of H_2S production by lead acetate strips were reported in Table 2, in which arbitrary values were assigned in function of the degree of blackening of lead acetate strips. The value ranged from 0, assigned to the strains producing very small amount of H_2S with the strips remaining white

Table 3. Conventional parameters and main secondary compounds concentrations of wines elaborated at cellar scale with indigenous (M3-59) and commercial starter (CS). The concentration was expressed as: [a] = g l^{-1}, [b] = %v/v; [c] = mg l^{-1}.

Parameters	M3-59	CS
Total acidity[a]	7,9	6,6
Volatile acidity[a]	0,40	0,42
Glucose + fructose[a]	0,0	0,0
Ethanol[b]	13,81	13,44
Malic Acid[a]	2,37	1,17
Lactic acid[a]	0,33	1,05
Acetaldehyde[c]	16,93	18,25
Ethyl acetate[c]	24,30	26,82
N-propanol[c]	28,75	27,78
Isobutanol[c]	30,57	59,88
D-amyl alcohol[c]	97,32	165,19
Isoamyl alcohol[c]	145,58	322,60

or near white, to 4, assigned to the strains producing very high amount of H_2S with strips very black. Different behaviours were found, with strains producing very small amounts of H_2S, such as M1-47, M1-55 and M3-60, and strains producing a considerable amount of this sulphur compound, such as M1-101, M1-104 and M1-107.

3.3. Inoculated fermentations at pilot scale

On the basis of these results, the strain M3-59 was selected for fermentation at pilot scale as this indigenous strain possessed the following suitable oenological characteristics: high resistance to antimicrobial compounds and medium H_2S production (technological group 13, Table 1); high fermentation rate (Fig. 3) and balanced production of secondary compounds (Table 2). This strain was tested in comparison to the commercial strain, commonly used in the cellar. Both the starters displayed ability to exhaust must sugars and to dominate the fermentation, with a strain implantation of 100% in both the fermentations. The other conventional parameters were very similar in the two wines and were comprised in the acceptable levels. High differences between the two wines were found for the content of amyl alcohols, which was very high in the wine obtained by inoculating the commercial starter. In particular, the content of isoamyl alcohol in the wine by commercial starter was 322 mg l^{-1}, a value slightly higher than 300 mg l^{-1}, the level considered negative for wine aroma.

4. Conclusions

This study was focused on the biodiversity of *S. cerevisiae* yeasts isolated from spontaneously fermenting grapes, collected from a specific production area of "DOC Matera" wine. It has to be underlined that studies on yeasts isolated from this environment had not been performed before.

The characterization for traits of oenological interest revealed that some of these strains possess interesting technological traits and could represent starter cultures available for winemakers, who are addressed to production of quality premium wines maintaining the differential properties of their own area. Different indigenous strains were characterized by fermentative performance similar or superior to commercial starter utilized by the wine cellar. The fermentation at pilot scale confirmed that the selected indigenous strain possesses oenological properties comparable with commercial starter; furthermore, the wine produced by the indigenous starter was characterized by a more balanced aroma than the wine produced by commercial starter.

Further experiments are in progress in order to test other indigenous strains as starter culture in real winemaking conditions. The strain M1-47 seems to be of interest for winemaking, being non H_2S producing, but further studies are necessary in order to test its behaviour at cellar level.

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