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Pilot-scale evaluation the enological traits of a novel, aromatic wine yeast strain obtained by adaptive evolution

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ABSTRACT

In the competitive context of the wine market, there is a growing interest for novel wine yeast strains that have an overall good fermentation capacity and that contribute favorably to the organoleptic quality of wine. Using an adaptive evolution strategy based on growth on gluconate as sole carbon source, we recently obtained wine yeasts with improved characteristics in laboratory-scale fermentations. The characteristics included enhanced fermentation rate, decreased formation of acetate and greater production of fermentative aroma. We report an evaluation of the potential value of the evolved strain ECA5™ for winemaking, by comparing its fermentation performance and metabolite production to those of the parental strain in pilot-scale fermentation trials, with various grape cultivars and winemaking conditions. We show that the evolved strain has outstanding attributes relative to the parental wine yeast strain, and in particular the production of less volatile acidity and greater production of desirable volatile esters, important for the fruity/flowery character of wines. This study highlights the potential of evolutionary engineering for the generation of strains with a broad range of novel properties, appropriate for rapid application in the wine industry.

1. Introduction

The wine market is currently facing several major challenges, including international competition, climate change and changing consumer preference. This context results in an increased dependence on technological innovation. As part of this, the development of new wine yeast strains with improved characteristics is now an important issue for the wine industry. Over the last two decades, metabolic engineering strategies based on recombinant DNA technologies have been extensively used, resulting in the development of wine yeast strains with improved fermentation and processing abilities, and with capacities for increasing the organoleptic quality of wine (Dequin, 2001; Pizarro et al., 2007; Schuller and Casal, 2005). However despite the successful generation of strains by genetic engineering, the poor consumer acceptance of GMO strains is a major obstacle to the use of these strains for winemaking. Due to these concerns, non GMO strategies, such as classical breeding or adaptive evolution approaches are becoming strategies of choice for the development of novel wine yeast strains.

Adaptive evolution, also called evolutionary engineering, is based on long-term cultivation under conditions in which specific selective pressure is applied. This favors the emergence of genetic variation, which can be followed by adaptive evolution of the yeast population and selection of variants with a desired phenotype. Using this approach, we recently obtained evolved strains from a commercial, diploid heterozygous wine yeast strain. The strains grew better than the parental strains on gluconate, a substrate metabolized through the pentose phosphate (PP) pathway (Cadière et al., 2011). These evolved strains exhibited marked changes in central carbon metabolism; in particular carbon was redirected toward the PP pathway. They also exhibited several novel, potentially valuable, characteristics for winemaking, that have been characterized in lab-scale wine fermentation conditions (Cadière et al., 2011). These traits include, relative to the parental strain, higher fermentation rate, reduced acetate production and enhanced formation of higher alcohols (also called fusel alcohols) and esters, which are major aroma compounds in wine (Lambrechts and Pretorius, 2000).

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Higher alcohols can have both positive and negative impacts on the aroma and flavor of wine depending of their concentration (Swiegers and Pretorius, 2005). They originate from α-keto acids (sugars metabolism) or from amino acids catabolism by the Ehrlich pathway (Hazelwood et al., 2008). At concentrations below 300 mg/L, they add a desirable level of complexity to wine, whereas at concentrations exceeding 400 mg/L, they have a detrimental effect (Swiegers and Pretorius, 2005). Ester compounds, including the acetate (isoamyl acetate, isobutyl acetate) produced from higher alcohols, and the ethyl esters (ethyl acetate, ethyl butyrate, ethyl hexanoate) produced from lipids and acetyl CoA metabolism, are desirable aroma compounds that give fruity flavors.

Lab-scale wine fermentations in small fermenters are useful for preliminary studies, but do not reproduce commercial wine fermentations perfectly. The results obtained with synthetic must (Bely et al., 1990) cannot reflect the complexity and the high variability of grape juice composition. Indeed, many variables are known to affect fermentative aroma including temperature, dissolved oxygen or yeast assimilable nitrogen (YAN) (Hazelwood et al., 2008). The nature and the availability of YAN have a great impact on both fusel alcohols and esters profiles during fermentation (Swiegers and Pretorius, 2005; Carrau et al., 2008). On the other hand, technological practices such as must clarification may affect yeast metabolism. Clarification consists of separating (by racking for example) the clear juice from the solid particles before alcoholic fermentation. During wine fermentation, the absence of oxygen prevents the biosynthesis of unsaturated fatty acids and sterols, which have important roles in protecting the yeast against ethanol stress (Alexandre et al., 1994). Therefore, yeast needs to obtain fatty acids from the medium. Clarification removes suspended solids, which contain the principal lipid supply for yeast cells. Consequently, extremely clarified juices lead to stuck or sluggish fermentation and may also lead to higher acetic acid concentrations in the wine.

Also, the conditions of small-scale fermentations tend to be poorly controlled and the hydrodynamic conditions differ from those in large volumes. Recent comparisons of lab and pilot scale fermentations revealed that scale does indeed affect various environmental parameters, in particular oxygen availability, and that there are small but noticeable differences in the kinetics and the production of aromatic molecules between these scales (Casalta et al., 2010; Rossouw et al., 2012). Therefore, although the results of lab-scale wine fermentation can be considered generally to reflect the winemaking process, a pilot-scale characterization is necessary to obtain a more rigorous and exact assessment of the potential of strains, especially those with new features.

In this study, we compared the main enological important traits of the evolved strain ECA5™ (Cadière et al., 2011) to those of the parental wine yeast strain in pilot-scale winemaking fermentation, in different grape juices and clarification conditions. We focused on fermentation performances and on the production of important by-products, including volatile acidity, esters and higher alcohols. We report evidence that the evolved strain has better properties than its parental strain Lalvin EC1118®: in particular it produces less acetate (volatile acidity) and more esters that are important for the fruity character of wine. These findings confirm the potential of evolutionary engineering approaches for generating strains with novel technological traits that can be used in the short-term in the winemaking industry.

2. Material and methods

2.1. Yeast strains

The *Saccharomyces cerevisiae* strains used in this study are the commercial strain Lalvin EC1118® and ECA5™, obtained by adaptive evolution of EC1118® (Cadière et al., 2011, patent FR 09/05585, 20/11/2009). Lalvin EC1118® used for pilot-scale experiments was obtained under the form of active dry yeasts. ECA5™ was produced as active dry yeast by Lallemand SA for the purposes of this study. Tanks were inoculated with 20 g/HL of dry active yeast, rehydrated according to the procedure recommended by the manufacturer.

2.2. Laboratory-scale fermentation at constant CO_2 production rate

MS synthetic medium that simulates standard grape juice was used for fermentations in 1 L fermentors (working volume) equipped with fermentation locks (Bely et al., 1990). The MS medium used contained 240 g/L of sugar (120 g/L of glucose and 120 g/L of fructose), 6 g/L malic acid, 6 g/L citric acid, and a nitrogen source composed of 28 mg/L nitrogen from ammonium and 96.3 mg/L from amino acids. To fulfill the lipid requirement of yeast cells during anaerobic growth, MS medium was supplemented with 7.5 mg/L ergosterol, 0.21 g/L Tween 80, and 2.5 mg/L oleic acid. The pH of the medium was 3.3. Once the maximal CO_2 production rate was reached (i.e. after a production of 5 g/L CO_2), the CO_2 production rate was feedback controlled at a constant rate by adding a concentrated solution (5 g/L) of assimilable nitrogen (di-ammonium phosphate) using a peristaltic pump (Manginot et al., 1997).

2.3. Pilot-scale fermentations

Fermentations at pilot scale were run in 100 L stainless steel tanks, described by Aguera and Sablayrolles (2005), at the fermentation temperatures indicated in Table 1. Most fermentation were run using grape musts from the South of France (Table 1). The grape musts were flash pasteurized and stored under sterile conditions (Aguera and Sablayrolles, 2005). CO_2 production was determined using a Brooks 5810 TR series gas flowmeter (Brooks Instrument, PA, USA), as described by Aguera and Sablayrolles (2005). There is a direct correlation between CO_2 production, sugar degradation and alcohol production (El Haloui et al., 1989).

2.4. Oxygen addition

Dissolved oxygen was added during fermentation to limit the risk of 'stuck' fermentation, as described by Blateyron (Blateyron et al., 1998). Fermenting grape must was pumped through a circulation loop at a flow rate of 2000 L/h. Pure oxygen was injected into this loop at a flow rate of 5 L/h through a stainless steel diffuser (N10A; Air Liquide, Toulouse, France). A transfer of 7 mg/L oxygen was obtained after 6.5 min of oxygen injection pumping, as determined by overall volumetric oxygen transfer calculations. The must was oxygenated when the ethanol concentration reached 3–4%, i.e. at the beginning of the stationary phase.

2.5. Post fermentation treatments

At the end of the fermentation, 50 mg/L SO_2 was added. Following tartaric stabilization, the wine was filtered and bottled. The end of the fermentation was determined from on line CO_2 measurements and by measurement of reducing sugar concentrations with dinitrosalicylic reagent, according to Miller (1959).

Table 1
Composition of grape musts and fermentation conditions.

Must	Sugar concentration g/L	Assimilable nitrogen mg/L	Turbidity NTU	Temperature
Chardonnay 07	220	216	6 and 150	18 °C
Chardonnay 08	223	152	140	18 °C
Macabeu 09	174	93	150	20 °C

Table 2
Metabolite and biomass levels and fermentation parameters of Lalvin EC1118 and ECA5 strains during wine fermentation.

	Chardonnay 07-180 NTU		Chardonnay 07-6 NTU		Chardonnay 08		Macabeu 09	
	Lalvin EC1118®	ECA5™	Lalvin EC1118®	ECA5™	Lalvin EC1118®	ECA5™	Lalvin EC1118®	ECA5™
Fermentation time (h)	245 ± 7	430 ± 0	477 ± 17	470 ± 0	320	320	175	200
V_{max} (g/L·h)	0.87 ± 0.14	0.98 ± 0.03	0.21 ± 0.01	0.26 ± 0.04	0.92	1.12	1.24	0.98
Population (106/ml)	82	72	50	40	90	90	99.8	101.8
Succinate (g/L)	0.58 ± 0.0	0.72 ± 0.0	0.43 ± 0.0	0.45 ± 0.0	0.89	1.04	0.53	0.69
Glycerol (g/L)	6.2 ± 0.1	6.5 ± 0.0	6.6 ± 0.1	6.9 ± 0.1	7.3	7.9	6.2	7
Volatil acidity (g/L H2SO4)	0.41 ± 0.01	0.12 ± 0.01	0.57 ± 0.0	0.43 ± 0.02	0.4	0.1	0.28	0.16
Ethanol (%vol)	14.1 ± 0.0	14.0 ± 0.0	14.1 ± 0.1	13.9 ± 0.2	14.05	13.9	11.1	11.2
Isobutanol (mg/L)	22.5 ± 0.7	32.23 ± 1.5	13.9 ± 0.4	20.8 ± 0.8	24.2	27.4	16.3	23.1
Isoamyl alcohol (mg/L)	207.3 ± 6.9	250.4 ± 8.8	160.4 ± 4.6	198.7 ± 6.5	182.6	226.5	123.3	153.6
Isobutyl acetate (mg/L)	0.0 ± 0.0	0.5 ± 0.0	0 ± 0	0.3 ± 0.0	0.1	0.3	0.09	0.25
Isoamyl acetate (mg/L)	7.2 ± 0.1	33.6 ± 1.9	4.8 ± 0.3	20.0 ± 1.6	3.2	10.4	3.2	7.1
Ethyl acetate (mg/L)	60.6 ± 1.6	118.5 ± 5.9	56.2 ± 1.8	114.2 ± 6.1	52.4	68.1	49.4	60.6
Ethyl butyrate (mg/L)	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.2	0.14	0.21	0.19
Ethyl hexanoate (mg/L)	0.3 ± 0.0	1.5 ± 0.1	1.0 ± 0.1	1.0 ± 0.2	0.24	0.51	0.41	0.6

Fermentations were considered to be complete when the residual sugar concentration was below 2 g/L.

2.6. Analytical methods

2.6.1. Cell counts

Cells were counted with a Coulter Z2 electronic counter (Coulter Multisizer 3, Beckman Coulter, Margency, France), fitted with a 100-μm aperture probe.

2.6.2. Metabolites determination

Glycerol, acetate and succinate concentrations were determined by HPLC (HPLC 1100, Agilent Technologies, Santa Clara, California) on a HPX-87H Aminex column (Biorad, Hercules, California). Glucose and fructose concentrations were determined enzymatically. Long-chain alcohols and esters were assayed by GC-HS, using a 125-7032E DBwax 30 m/0.53 mm/1 μm Agilent Technology column (Agilent Technologies, Santa Clara, California). The ethanol concentration was determined by measuring density, the volatile acidity by the bromophenol blue method, the SO_2 concentration by iodometry and total acidity by titration.

2.7. Sensory analysis

Twenty people were chosen as judges to form a panel for sensory analysis. Wines were evaluated by this untrained panel, on olfactory and taste attributes (Table 3) in monadic service, according to a random order (Latin square) minimizing carry-over effects. Judges rated each attribute on an unstructured linear scale from “low” to “high”. Scores were collected with a computerized data acquisition system (FIZZ software version 2.40 A, Biosystemes, Couternon-France).

Table 3
Concentration of aroma compounds usual in wine, and aroma descriptor.

	Concentration in wine (mg/L)	Aroma threshold (mg/L)	Aroma descriptor
Isobutanol	9.0–174	40*	Fusel, spirituous, bitter
Isoamyl alcohol	6.0–490	30*	Harsh, nail polish, whisky, malt
Isobutyl acetate	0.01–1.6	1.6**	Banana, fruity
Isoamyl acetate	0.1–8.4	0.03*	Banana, pear
Ethyl acetate	22.5–208	7.5*	Nail polish, fruity, pineapple
Ethyl butyrate	0.01–3.8	0.02*	Floral, fruity
Ethyl hexanoate	0.3–1	0.014*	Apple peel, fruit

*10% Ethanol, **synthetic wine.

2.8. Statistical analysis

Sensory data were converted by the FIZZ software into marks from 0 to 10. Data analysis was performed using XLSTAT software (2008 version, Addinsoft, France).

Patterns within the different sets of data were investigated by principal component analysis (PCA). PCA is a bilinear statistical modeling method for decreasing the number of variables and identifying relationships between variables. We used centered data and R Development Core Team software, with the specific package ade4 (Dray and Dufour, 2007).

3. Results and discussion

3.1. Nitrogen requirements

The nitrogen requirements of *S. cerevisiae* differ between strains. During winemaking fermentations, the fermentation rate increases during the growth phase, reaches a maximum value at the end of this phase, and progressively declines during the stationary phase, until complete sugar exhaustion. Manginot et al. (1997) proposed a test for estimating yeast nitrogen requirements during the stationary phase of wine fermentation; it consisted in determining the quantity of nitrogen required to maintain a constant fermentation rate during this phase. Using this test, we compared the nitrogen requirement of ECA5™ to those of 113 commercial wine yeasts. Reproducibility of results was tested by fermenting strains in triplicate. The strain ECA5™ exhibited the lowest requirement for nitrogen (Fig. 1); it was 40% less than the parental strain Lalvin EC1118®. This result is in good agreement with the results previously obtained in fermentation conditions with low nitrogen content (100 mg/L), high sugar concentration 240 g/L and low temperature (18 °C). Indeed, in these conditions, ECA5™ maintained a higher fermentation rate over time and complete sugar degradation 200 h earlier than the parental strain (Cadière et al., 2011). As low nitrogen level is a prevalent cause of slow fermentations (Blateyron and Sablayrolles, 2001), this property of ECA5™ may be valuable for the fermentation of grape musts with low nitrogen contents.

3.2. Characteristics of ECA5™ during wine fermentation at pilot scale

The properties of ECA5™ were compared to those of the parental strain under different wine fermentation conditions, in 100 L fermentation tanks. Three musts with different yeast assimilable nitrogen (YAN) were used: Chardonnay 07, Chardonnay 08

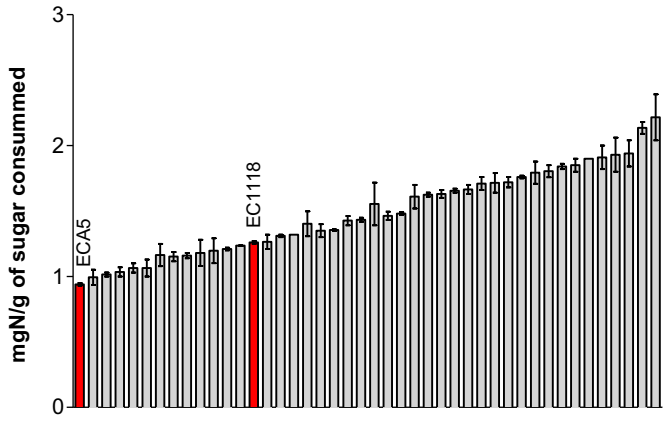

Fig. 1. Nitrogen requirement of ECA5™ and of 44 commercial wine strains. The nitrogen requirement was estimated from the amount of nitrogen necessary to maintain the fermentation rate at a constant value between 20 and 70 g/L of produced CO_2. This variable is expressed in mg of nitrogen per gram of sugar consumed.

and Macabeu 09. In addition, the effect of two different levels of clarification was studied on Chardonnay 07. Fermentation duplicates were performed for the two strains on Chardonnay 07, 6 and 180 NTU and were highly reproducible: the relative standard deviations relating to the fermentation time and the maximal fermentation rate (V_{max}) were below 16% (Table 2). We first showed that the fermentation performances and the total population of ECA5™ were close to those of Lalvin EC1118® under all conditions. These data contrast with the better performance observed for the evolved strain compared to Lalvin EC1118® in laboratory conditions (Cadière et al., 2011).

3.3. Formation of aromatic compounds

At high initial nitrogen concentration, both strains produce higher concentrations of higher alcohols and of esters. Such observation is in line with previous studies, although the relationship between initial nitrogen concentration and aroma production may differ depending on the strain (Carrau et al., 2008). The evolved strain ECA5™ produced 1.2–1.5-fold more higher alcohols than Lalvin EC1118® in all conditions (Table 2). Although the absolute value of 2-phenylethanol was not determined in this study, on line GC–MS monitoring of major aroma compounds in Macabeu revealed that the level of this compound was 1.7-fold higher in ECA5™ than in EC1118®. An even greater impact, between 2.2 and 4.6 was observed for the esters from higher alcohols, especially isobutyl acetate, isoamyl acetate in all musts (Table 2) and phenyl acetate (1.4-fold more, monitored in Macabeu). The amount of isobutyl acetate produced by the evolved strain was nevertheless in the usual range found in wines. In contrast, the amount of isoamyl acetate was between 1.2 and 1.8 fold higher than the highest usual concentration.

ECA5™ produced more ethyl acetate and ethyl hexanoate than the other strains. Ethyl acetate at high concentration is associated with bad flavors, but in the case of ECA5™, the production of ethyl acetate was still in the concentration range usually found in wines (Table 3). ECA5 produced between 2 and 5-fold more ethyl hexanoate (except in clarified must), a compound associated with floral and fruity descriptors.

These data show that strain ECA5™ produced larger amounts of volatile compounds than the parental strain, irrespective of fermentation conditions and must composition. The significant higher production of higher alcohols by ECA5™ suggests a less efficient use of α-keto acids for the synthesis of amino acids, a higher glycolytic flux or a more efficient amino acids catabolism than the parental strain. On the other hand, the synthesis of esters – acetates produced from higher alcohols and ethyl esters – was markedly increased, which may result from increased synthesis of higher alcohols and/or reflect changes in acetyl CoA metabolism. Indeed, ECA5™ produces less acetate and has a higher flux toward lipid biosynthesis than Lalvin EC1118® (Cadière et al., 2011), which is consistent with its greater production of ethyl esters.

3.4. Global analysis of properties of wine issued from ECA5™ fermentation

PCA was used to integrate the various properties of wine issued from ECA5 fermentation. We included the variables that differed most between the strains, namely: isoamyl alcohol, isoamyl acetate, isobutanol, isobutyl acetate, ethyl acetate, ethyl hexanoate, ethyl butyrate, volatile acidity, glycerol and succinate levels. In the resulting PCA plot, the data points are separated along a curve running across the first two principal components, which account for 88.2% of the variance (Fig. 2A). The Dim1 axis (60.22% of the variance) mostly corresponds to isoamyl alcohol, isoamyl acetate, isobutanol, isobutyl acetate, ethyl acetate, ethyl hexanoate, ethyl

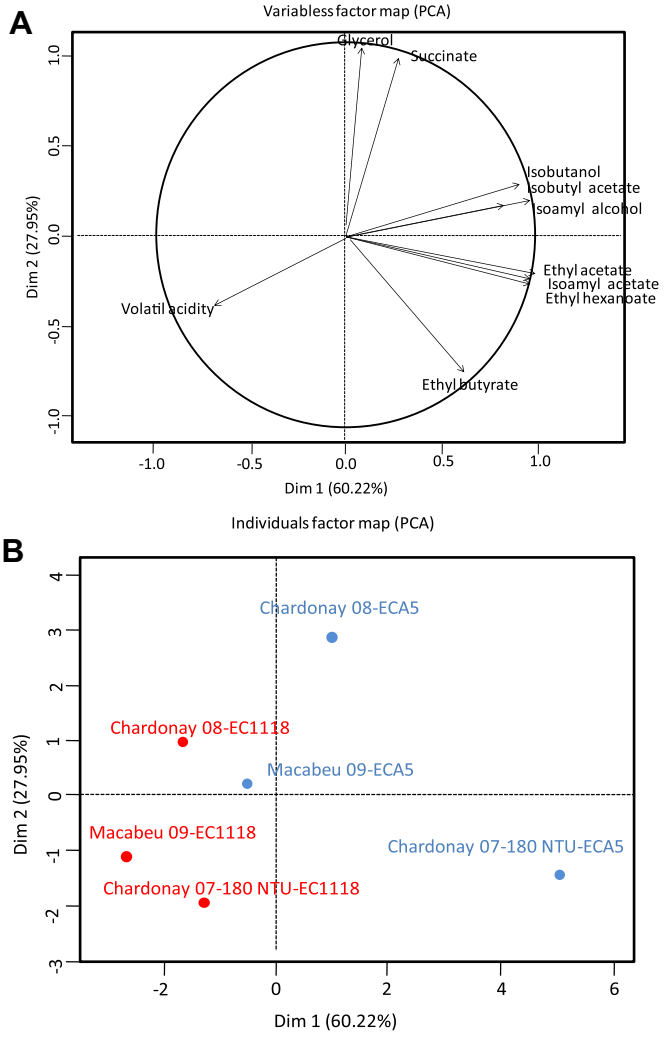

Fig. 2. PCA analysis: global analysis of properties of wine issued from ECA5™ fermentation. The variables used for PCA analysis were isoamyl alcohol, isoamyl acetate, isobutanol, isobutyl acetate, ethyl acetate, ethyl hexanoate, ethyl butyrate, volatile acidity, glycerol and succinate levels.

butyrate, volatile acidity, on the one hand, and the Dim2 (27.95%) axis to succinate and glycerol on the other hand.

Fig. 2B shows the projection of the strains in the two-dimensional space defined by the Dim1 and Dim2 axis. Strains were clearly separated on the basis of their production of aroma compounds and volatile acidity on the first axis, which can be interpreted as overproduction of higher alcohols and esters by ECA5™ whatever the composition in nitrogen.

On the second axis, groups were separated as a function of succinate and glycerol production. These axes explain the characteristics of the wine issued of the fermentation of ECA5™ on chardonnay 08 must. Therefore, for these traits, the most important effect is the must, not the strain.

3.5. Impact of clarification on the fermentative properties of ECA5™

ECA5™ is a low acetate producer in synthetic medium (Cadière et al., 2011), so we assessed the potential of this strain for the fermentation of clarified musts. Fermentations were performed in 100 L fermentation tanks, in duplicate, in two conditions of clarification. The degree of clarification was determined by measuring the turbidity and is expressed in nephelometric turbidity units or NTU. Chardonnay must 07 (Table 2), at two levels of clarification, 150 NTU (standard level) and 6 NTU (extreme conditions), was used and fermentations were performed at 18 °C.

In extremely clarified must, the production of volatile acidity (which is mainly constituted of acetate) by Lalvin EC1118® and ECA5™ was 1.4 and 3.6 times that in less clarified must, respectively, and was 30% less for ECA5™ than Lalvin EC1118® (Table 2). In extremely clarified must, there was only low level production of isobutanol, isoamyl alcohol and isoamyl acetate. The concentrations of these aroma compounds were reduced similarly for both strains (1.5 times for isobutanol and isoamyl acetate, 1.3 times for isoamyl alcohol) (Table 2). Thus, in both clarification conditions, the ECA5™ strain consistently produced less volatile acidity and more volatile compounds than the parental strain.

3.6. Sensorial analysis

The particular aromatic profile of ECA5™ prompted us to perform a sensorial evaluation of the wines produced. The four wines (Lalvin EC1118® 150 NTU and 6 NTU and ECA5™ 150 NTU and 6 NTU) were evaluated by a panel of 20 judges for their olfactory and gustatory characters and for their global quality.

The first axis, representing 59.8% of the total variance, was loaded positively with the sweetness and pear attributes and negatively with citrus aroma. Bitterness and vegetable contributed to the second axis, which explained 25.6% of the variance. Sweet and amylic (banana) characters were correlated to the global quality (Fig. 3). The preferred wines were those fermented by ECA5™ strain and the best score was for the wine fermented by ECA5™ from the 6 NTU must.

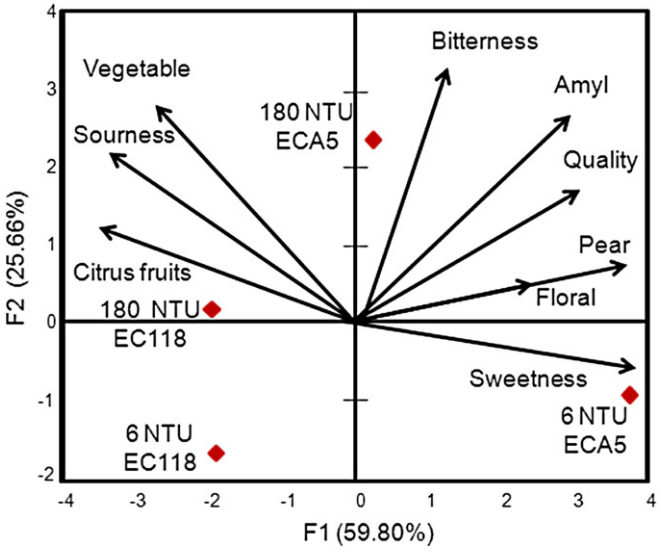

Fig. 3. PCA analysis: contribution of the variables and distribution of wines. The variables used for PCA analysis were sweetness, pear attributes, citrus aroma, the bitterness, the vegetable and the amylic characters, and global quality.

4. Conclusion

We report here a detailed characterization, in pilot-scale fermentation trials, of ECA5™, a yeast strain recently obtained by evolutionary engineering, compared to its parental strain. This study highlights the novel technological traits of this evolved strain and confirmed a number of observations previously made at the laboratory scale in synthetic must (Cadière et al., 2011). We first showed that ECA5™ has outstanding properties regarding the use of nitrogen, with the lowest requirement compared to 113 commercial strains used for winemaking. ECA5™ has therefore a very high potential for the fermentation of low nitrogen grape musts, which often lead to stuck fermentations. Another interesting trait of ECA5™ is the low production of volatile acidity, with confirms its potential value for the fermentation of highly clarified grape musts. The most remarkable property of ECA5™ is its capacity to overproduce esters, including higher alcohol acetates and ethyl esters. As these aroma compounds are responsible of various floral and fruity notes (peach, pear, banana, pineapple…), this makes ECA5™ a very attractive strain for enhancing the aromatic value of grape juices. More generally, this study illustrates the power of adaptive evolution strategies to generate diversity and to produce strains that can be rapidly used in the industry.

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