Influence of nutrients and temperature on the production of fermentative aromas in winemaking conditions by an evolved wine strain of Saccharomyces cerevisiae

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Introduction

Alcoholic fermentation is a key step in oenology. Its conduct has a direct impact on the quality of the wine and the wine sector shows a growing interest in approaches to better understand and control the fermentation depending on the desired type of wine. Fermentative aromas (esters and higher alcohols) play an important role in the organoleptic profile of young wines. Their production depends both on yeast strain and on fermentation conditions.

Currently, the online monitoring of fermentations focuses on the main reaction of the alcoholic fermentation, i.e. the bioconversion of sugar into ethanol and CO_2. With some sensors, especially those measuring the release of CO_2, we can not only estimate the concentrations in alcohol and residual sugar but also the instantaneous fermentation rate (1). This latter information is very important because the instantaneous fermentation rate is directly proportional to (i) the activity of micro-organisms (microbiological significance) and (ii) the energy required for temperature control (technological importance).

The next challenge is to act directly on the organoleptic characteristics of the wine. The recent development of an online monitoring tool of aroma molecules has allowed us to accurately determine the kinetics of production of these volatile compounds (2). The objective of the current study is to use this innovative device to determine the combined impacts of nutrients (nitrogen, lipids) on the most abundant fermentative aromas. We studied the production of these volatile compounds by two *Saccharomyces cerevisiae* strains having different aromatic profiles: Lalvin EC1118® and Affinity™ ECA5, obtained by evolutionary engineering of Lalvin EC1118® and presenting an enhanced aroma production (3).

Material & Methods

Fermentations were performed using two commercial wine yeast strains: Lalvin EC1118® and Affinity™ ECA5, obtained by evolutionary engineering of Lalvin EC1118®. In these culture media, the lipid fraction was represented by a commercial mixture of phytosterols (mainly β-sitosterol, campesterol and stigmasterol) similar to the sterols present in grape solids.

First, fermentations were performed in 330 ml fermenters in synthetic media having variable initial concentrations of nitrogen (70, 200 and 330 mgN/l) and lipids (2, 5 and 8 mg/l) at three different temperature (20, 24 and 28°C).To determine the effects of temperature, initial nitrogen and lipid contents on the production of volatile compounds, a response surface methodology was applied with a Box-Behnken experimental design (4). The effects of these three factors were studied in a single block of 16 sets of test conditions and 4 central points. The order of the experiments was fully randomized. Statistical analysis was performed with R software, version 2.14.2. A quadratic polynomial model was defined to fit the response.

Then, the fermentations were made in 10 l tanks at two nitrogen (70 and 330 mgN/l) and phytosterol (2 and 8 mg/l) contents at 24°C. The release of CO_2 (corresponding to the fermentation kinetics) was measured using a mass flow meter. The concentrations of volatile compounds in the headspace of the fermenter were measured with an online GC (gas chromatography) device: the head space gas was pumped through heated transfer lines. Carbon compounds were concentrated in a cold trap and analyzed by GC (Figure 1).

Results & Discussion

– Relative performances of Affinity™ ECA5 compared to Lalvin EC1118®

In previous studies (3, 5, 6), the capabilities of Affinity™ ECA5 and of its parental strain Lalvin EC1118® were compared and major differences were identified. Especially, the production of fermentative aromas was enhanced for the evolved strain (3).
In this work, to evaluate the genericity of these observations, the productions of higher alcohol and esters of the two yeast strains were compared in various fermentation conditions. Thanks to a Box-Behnken design (4), we evaluated combined effects of three environmental parameters (nitrogen, phytosterols and temperature) varying in broad ranges. For each aroma compound, we plotted the ratio of final concentration obtained with the strain Affinity™ ECA5 divided by the one obtained for Lalvin EC1118® on the figure 2. These ratios were systematically greater than 1 for higher alcohols (except propanol) and acetate esters: these molecules were systematically overproduced by the evolved strain whatever the conditions of fermentation. These molecules were thus identified as metabolic markers of the evolved yeast strain. Conversely, we found that for the ratios for the fatty acids and ethyl esters, 1 was included in the confidence interval; thus these compounds were not systematically overproduced by Affinity™ ECA5.

– Kinetics profiles of fermentative aromas

We compared the kinetic profiles of production of fermentative aromas of the evolved and ancestral strains using the online GC system.
First, we observed an interaction between nitrogen and phytosterol content for both strains; but this interaction was more marked in the media containing 330 mg/l of assimilable nitrogen and for Affinity™ ECA5. This interaction resulted in differences in the kinetic profiles of synthesis of aroma compounds. The effect of the medium composition and the performance of each strain were visible on both the total production of volatile compounds and on their kinetics of synthesis (Table 1, Figure 3).
Here, we presented only the example of the specific rate of production of isoamyl acetate (Figure 3). The shapes of these specific rates were dependent on the strain but also on the environmental parameters (Figure 3). At high nitrogen content, the profiles of the specific rates of acetate ester production were governed by the lipid dose, showing the predominance of the environmental effects. At 2 mg/l of lipids, the specific production rate peaked very quickly and suddenly decreased, whereas at 8 mg/l of phytosterols, once the maximum value was reached the decrease was much slower (Figure 3). The factors that affected the flux of ester synthesis seemed to be the same for both strains. Conversely, in SM70, the strain effect was dominant because it was the capability of each strain to respond to stress that seemed to impact the flux of ester production.Finally, when comparing the maximal values of total production and of specific rate of these esters, it was surprising to observe that the ranking of the fermentation conditions according to these two parameters was different (Figure 3, Table 1). The study of these specific rates was interesting to better understand the regulation of the metabolic pathways of aroma production.

Table 1 Total production (mg/l) of fermentative aromas by Lalvin EC1118® and Affinity™ ECA5

Initial nitrogen concentration (mg/l)	Initial phytosterol concentration (mg/l)	Propanol		Isobutanol		Isoamyl alcohol	
		Total production (mg/l) by EC1118	Total production (mg/l) by ECA5	Total production (mg/l) by EC1118	Total production (mg/l) by ECA5	Total production (mg/l) by EC1118	Total production (mg/l) by ECA5
70	2	5.04	4.52	25.8	36.1	165	276
70	8	5.25	4.63	28.8	36.5	175	270
330	2	25.6	22.2	19.9	42.1	103	148
330	8	27.6	21.2	36.3	54.9	156	221
Initial nitrogen concentration (mg/l)	**Initial phytosterol concentration (mg/l)**	**Ethyl acetate**		**Isobutyl acetate**		**Isoamyl acetate**	
		Total production (mg/l) by EC1118	**Total production (mg/L) by ECA5**	**Total production (mg/l) by EC1118**	**Total production (mg/L) by ECA5**	**Total production (mg/l) by EC1118**	**Total production (mg/l) by ECA5**
70	2	37.7	27.1	0.05	0.23	0.45	3.29
70	8	39.7	22.9	0.05	0.19	0.43	2.14
330	2	67.7	124	0.36	1.79	3.83	13.0
330	8	52.8	73.3	0.31	1.45	2.87	12.0
Initial nitrogen concentration (mg/l)	**Initial phytosterol concentration (mg/l)**	**Ethyl hexanoate**		**Ethyl octanoate**			
		Total production (mg/l) by EC1118	**Total production (mg/L) by ECA5**	**Total production (mg/l) by EC1118**	**Total production (mg/l) by ECA5**		
70	2	0.67	0.72	0.64	0.62		
70	8	0.63	0.68	0.57	0.55		
330	2	1.32	1.71	1.71	2.05		
330	8	0.88	1.07	1.26	1.18		

– Bioconversion between higher alcohol and its acetate ester

We wondered if the systematic overproduction of acetate esters by the evolved strain was solely due to the overproduction of their higher alcohol precursor or if the activity of the alcohol acetyltransferases (Atf1p and Atf2) responsible of this bioconversion was also involved. Indeed, the difference in production of these esters between the two strains was more important than that of the higher alcohols.

We studied two couples of higher alcohol / acetate ester: isobutanol and isobutyl acetate; and isoamyl alcohol and isoamyl acetate. For these two couples and for both strains, the conversion yield was dependent on the initial nitrogen and phytosterol contents: the highest yields were obtained at high nitrogen content (330 mg/l) and low phytosterol content (2 mg/l) (Figure 4). For Lalvin EC1118®, the yield remained constant throughout the fermentation. By contrast, for Affinity™ ECA5, there were generally two production phases, with a much higher yield in the second phase. The transition occurred during the stationary phase and was particularly visible with 330 mg/l of nitrogen and 8 mg/l of phytosterols (Figure 4). A possible hypothesis to explain this drastic change can be related to the presence of lipids, which are known to repress the expression of *ATF1* (7). Thus, we realized some phytosterol additions at different stages of the fermentation to confirm the impact of this factor. These additions had no impact on the

bioconversion of higher alcohol into acetate ester by Lalvin EC1118® (Figure 5): the bioconversion yields remained constant despite the additions. On the other side, the additions of phytosterols dramatically lowered the bioconversion of higher alcohols for Affinity™ ECA5. Indeed, after each lipid addition, the yield was divided by two (Figure 5). This detailed study showed that this difference seemed to be related to a modified management of phytosterols by the evolved strain.

Conclusion

This dynamic study allowed us highlighting differences in the chronology of production of aroma compounds between the two strains, which suggest modifications in the regulation of aroma synthesis. One of the key findings was the impact of phytosterols on the production of aroma compounds for the two strains. We noted substantial differences in the management on the lipid source for the evolved strain. These metabolic modifications were particularly visible on the bioconversion of higher alcohols into acetate esters.

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