Dynamics and quantitative analysis of the synthesis of fermentative aromas by an evolved wine strain of *Saccharomyces cerevisiae*

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Abstract

We performed a dynamic and quantitative analysis of the synthesis of fermentative aromas by an aromatic wine yeast (ECA5) obtained by adaptive evolution. During fermentation at pilot scale on synthetic and natural musts, ECA5 produced volatile compounds (higher alcohols and their acetates, ethyl esters) at higher rates than the ancestral strain, with the exception of propanol. Marked differences in the chronology of synthesis of several compounds were observed between the two strains. Overproduction of phenyl ethanol occurred mainly during the growth phase for ECA5, consistent with its higher flux through the pentose phosphate pathway, which plays a key role in biosynthetic processes. The kinetics of production of isobutanol and isoamyl alcohol were differently affected by different media (synthetic or natural must), and in particular according to the nature of the sterols in the media (ergosterol or phytosterols). We also observed differences in the chronology of synthesis of ethyl acetate and isoamyl acetate or ethyl esters, suggesting that the regulation of the synthesis of these compounds in the evolved strain differs from that in the ancestral strain. This study shows that a dynamic analysis of volatile compounds, using high acquisition frequency online gas chromatography, can provide novel insights into the synthesis and regulation of aroma, and is thus a potentially powerful tool for strain characterization.

Keywords

Wine yeast; adaptive evolution; online monitoring; gas-liquid balance; higher alcohols; esters.

1. Introduction

Wine aroma is one of the principal attributes determining the preferences of wine consumers (Lee and Noble, 2003; Swiegers *et al.*, 2005; Ugliano *et al.*, 2010). Most fruity aroma compounds, including esters in particular, are produced by yeast during alcoholic fermentation. Several studies have assessed the influence of fermentation parameters (principally temperature and nitrogen addition) on the final fermentative aroma content of the wine, focusing mostly on higher alcohols and esters (Swiegers *et al.*, 2005). The choice of yeast strain is also a determinant of the final concentration of these volatile compounds (Camarasa *et al.*, 2011; Molina *et al.*, 2009, Torija *et al.*, 2003). Therefore, the development of new wine yeast strains with improved aromatic production properties is an important issue for the wine industry. Given the poor consumer acceptance of genetically modified organisms (GMO) in the food industry, GMO-free strategies, such as experimental evolution, have recently received much attention for improving wine yeast traits (Cadiere *et al.*, 2011; Kutyna *et al.*, 2012; Tilloy *et al.*, 2014). Adaptive evolution involves maintaining yeast during a large number of generations in a controlled, selective environment. Strains with mutations having beneficial impact under the conditions used will increase in frequency in the population, and can then be selected as evolved variants.

Using this approach and gluconate as sole carbon source, we previously selected an evolved wine yeast (Affinity ECA5^{TM}) with increased flux through the pentose phosphate pathway. This strain displays several novel traits that are potentially beneficial for winemaking (Cadiere *et al.*, 2011, 2012; patent FR 09/05585, 20/11/2009). In particular, it produced markedly less volatile acidity but greater amounts of esters, responsible of various floral and fruity notes, than the ancestral

strain; these characteristics make ECA5 an attractive strain for enhancing the aromatic value of grape juices.

Although the amounts of esters and higher alcohols were consistently greater in the final product of ECA5 fermentation than the ancestral strain fermentation, the dynamics of synthesis of these compounds has not been compared between wine fermentations with the two strains. As wine fermentation involves a short growth phase followed by a long, nitrogen-limited stationary phase, the synthesis of aroma compounds varies greatly during the fermentation process.

The objective of this study was to compare the kinetics of production of the main fermentative aromas by ECA5 and its ancestral strain EC1118, in natural and synthetic musts, at pilot-scale, using a recently developed online monitoring system. The high acquisition frequency of online gas chromatography (GC) makes it possible to determine kinetic parameters, and to calculate the rates of synthesis for volatile compounds (Mouret *et al.*, 2014a). We also determined the gas-liquid balances of aroma production to take losses into account and to distinguish yeast metabolic synthesis from physicochemical effects (Morakul *et al.*, 2013). This dynamic and quantitative analysis of the synthesis of major aroma compounds by the evolved and ancestral strain was expected to elucidate the metabolic basis of the evolved phenotype. In both synthetic and natural grape musts there was a marked overproduction of acetate esters by the evolved strain. Also, there were differences in the regulation of the production of several aroma compounds between the evolved and ancestral strains. Furthermore, we also found differences in the kinetics of synthesis of some aroma compounds depending on the nature of sterols in the media.

2. Materials and Methods

2.1 Fermentation

2.1.1 Yeast strains and culture media

The *Saccharomyces cerevisiae* strains used in this study were the commercial strains Lalvin EC1118® and Affinity ECA5^{TM} (Lallemand SA, Montreal, Canada), obtained by adaptive evolution of EC1118® (Cadiere *et al.*, 2011, 2012; patent FR 09/05585, 20/11/2009). Media or musts in fermentation tanks were inoculated with 200 mg/l active dry yeast that had been rehydrated by incubation with 50 g/l glucose for 30 minutes at 35 °C.

In this study, we used a natural must and a synthetic culture medium. The natural must was harvested in 2009 in the South of France (Maccabeu), flash-pasteurized, and stored under sterile conditions. It contained 181 g/l of sugar and 243 mg/l of assimilable nitrogen. The synthetic medium was formulated to mimic grape musts (Bely *et al.*, 1990). It contained 120 g/l glucose, 120 g/l fructose, 6 g/l malic acid, 6 g/l citric acid, salts (0.75 g/l KH_2PO_4; 0.50 g/l K_2SO_4; 0.25 g/l $MgSO_4$; 0.155 g/l $CaCl_2$; 0.20 g/l NaCl), vitamins (20 mg/l myo-inositol; 1.5 mg/l pantothenic acid; 0.25 mg/l thiamine; 2 mg/l nicotinic acid, 0.25 mg/l pyridoxine; 0.003 mg/l biotin), trace elements (4 mg/l $MnSO_4$; 4 mg/l $ZnSO_4$; 1 mg/l $CuSO_4$; 1 mg/l KI; 0.4 mg/l $CoCl_2$; 1 mg/l H_3BO_3; 1 mg/l $(NH_4)_6Mo_7O_{24}$) and anaerobic factors (0.05% v/v Tween 80; 3.75 mg/l ergosterol; 0.0005% v/v oleic acid). Tartaric acid was replaced with citric acid to prevent the formation of tartrate precipitate during freezing at -20°C. The source of nitrogen was a mixture of ammonium (30%) and amino acids (70%); the

assimilable nitrogen concentration was 425 mgN/l. The amino-acid content of the medium was as follows: 44.7 mg/l aspartic acid, 123.0 mg/l glutamic acid, 145.3 mg/l alanine, 374.4 mg/l arginine, 13.1 mg/l cysteine, 505.3 mg/l glutamine, 18.3 mg/l glycine, 32.7 mg/l histidine, 32.7 mg/l isoleucine, 48.4 mg/l leucine, 17.0 mg/l lysine, 31.4 mg/l methionine, 38.0 mg/l phenylalanine, 612.6 mg/l proline, 78.5 mg/l serine, 75.9 mg/l threonine, 179.3 mg/l tryptophan, 18.3 mg/l tyrosine and 44.5 mg/l valine.

To study the effects of sterols on the synthesis of higher alcohols, fermentations were performed at 28°C in synthetic media containing 3.75 mg/l of either ergosterol or phytosterols (ref 85451, Sigma Aldrich) as lipid source. The composition of these media was the same as that of the synthetic medium described in the previous paragraph; it contained 100 g/l glucose, 100 g/l fructose and 250 mg/l assimilable nitrogen (the relative proportions of the different sources were the same as that in the medium containing 425 mg/l). A stock solution of 15 mg/l phytosterols (β-sitosterol 90%, campesterol 5% and stigmasterol 5%) was made up in Tween 80 and ethanol (1:1, v/v).

2.1.2 Cell population and metabolite determination

During fermentation, the cell population was determined with a Coulter Counter (model Z2, Beckman-Coulter, Margency, France) fitted with a 100 µm-aperture probe.

Volatile acidity (proportional to the concentration of acetic acid) was determined by the bromophenol blue method (Gallery, Thermo Fisher Scientific, Asnieres sur Seine, France).

2.1.3 Tanks and fermentation control

Fermentations were run at pilot scale, in 10-liter stainless steel tanks, at 20 °C. The amount of CO_2 released was measured accurately and automatically with a gas mass flow meter, for calculation of the rate of CO_2 production (dCO_2/dt).

Each pilot-scale fermentation was performed once. We previously determined that duplicate experiments run with this online monitoring system yield highly reproducible results: for all the various volatile compounds assessed, the relative standard deviation between duplicates was very low throughout the fermentation process (Mouret *et al.*, 2012, 2014a): 4% for propanol, 3% for isobutanol, 6% for isoamyl alcohol, 3 % for isoamyl acetate, 2% for ethyl hexanoate and 4% for ethyl octanoate.

2.2 Analysis of higher alcohols and esters

The concentrations of volatile compounds in the headspace of the fermentor were measured with an online GC device.

Headspace gas was pumped from the tank at a flow rate of 14 mL/min, through a heated transfer line. Carbon compounds were concentrated in a cold trap (Tenax TM) for 6 minutes, desorbed at 160 °C for 1 minute, and analyzed with a Perichrom PR2100 GC coupled to a flame ionization detector (Alpha MOS, Toulouse, France). The details of the GC method and the calibration procedure were as previously described by Mouret *et al.*, 2014a.

2.3 Volatile compound balances during fermentation

2.3.1 Concentrations in the liquid

The concentration of a volatile compound in the liquid ($C^{liq}(t)$) was calculated from the concentration measured online in the gas phase, expressed as $C^{gas}(t)$ in mg/l of CO_2, using the partition coefficient (k_i) value (Eq. 1).

$$C^{liq}(t) = \frac{C^{gas}(t)}{k_i} \qquad \textbf{Equation 1}$$

The value of k_i (Eq. 2) was calculated with the model developed by Morakul *et al.*, 2011, as a function of the fermenting must composition, characterized by ethanol concentration and temperature.

$$\ln k_i = F1 + F2 \times E - \frac{F3 + F4 \times E}{R}\left(\frac{1000}{T} - \frac{1000}{T_{ref}}\right) \qquad \textbf{Equation 2}$$

where E is the ethanol concentration (g/l) in the liquid phase, calculated from the measurement of the amount of CO_2 released, which is proportional to sugar consumption; T is the current absolute temperature, T_{ref} the absolute reference temperature (*i.e.* 293.15 K, or 20 °C, in this study). F1, F2, F3 and F4 are constants, identified for each volatile compound. The values of these parameters for the various molecules considered have been determined by Mouret *et al.*, 2014a, 2014b.

2.3.2 Losses in the exhaust gas

Losses into the exhaust gas were calculated with Eq. 3.

$$L(t) = \int_0^t C_{gas}(t) \times Q(t) \times dt \qquad \textbf{Equation 3}$$

where Q(*t*) is the CO_2 flow rate at time *t,* expressed in liters of CO_2 per liter of must and per hour.

2.3.3 Total production

The total production of a volatile compound at time *t*, expressed as P(*t*) in mg/l of must, was calculated by adding the concentration in the liquid phase, expressed as $C^{liq}(t)$ in mg/l of must, to the amount of the volatile compound lost in the gas phase, expressed as L(*t*) in mg/l of must (Eq. 4).

$$P(t) = C^{liq}(t) + L(t) \qquad \textbf{Equation 4}$$

This production value represents the capability of the yeast to produce a volatile compound, independent of the subsequent fate of the compound: accumulation in the liquid phase or evaporation.

2.3.4 Calculation of the rates and specific rates of production, accumulation in the liquid phase and loss into the gas phase

The high frequency of online GC measurements (up to one measurement per hour) made it possible to calculate the rates of volatile compound production, accumulation and loss, using sliding-window second-order polynomial fitting in a custom-developed Labview application.

The specific rates were calculated by dividing the corresponding rates by the cell count.

3. Results and discussion

We compared the kinetic profiles of production of fermentative aromas of the evolved and ancestral strains ECA5 and EC1118 in a natural grape must and a synthetic medium mimicking grape must, using an innovative online GC system. We monitored ten fermentative aroma compounds from various chemical families and having different flavor properties throughout the fermentation process: four higher alcohols (propanol, isobutanol isoamyl alcohol and 2-phenylethanol), four esters of higher alcohols (ethyl acetate, isobutyl acetate, isoamyl acetate and 2-phenylethylacetate) and two ethyl esters (ethyl hexanoate and ethyl octanoate).

We previously showed that losses into the exhaust gas are highly compound dependent: as expected, they are negligible for higher alcohols, but are substantial for esters (Morakul *et al.*, 2013; Mouret *et al.*, 2014a). For example, at 24°C, 0.61% of the isobutanol and 42% of the ethyl hexanoate are lost during the fermentation process (Mouret *et al.*, 2014a). Consequently, the amounts of higher alcohols produced by yeast can be accurately deduced from the liquid concentrations, but assessment of the metabolic capacity of yeast for the synthesis of esters requires assays of both the liquid and gas phases.

We therefore determined the total production of fermentative aromas (except for 2-phenylethanol and 2-phenylethylacetate) as the sum of liquid content and gaseous loss (Table 1).

To compare the chronology of synthesis between volatile compounds, we characterized the production profile of each in terms of various representative kinetic

measures (Tables 2a and 2b): (1) start of synthesis (defined as the percentage of sugar consumed at which the product concentration first exceeded 10% of its maximum concentration), (2) end of synthesis (defined as the percentage of sugar consumed at which the product concentration reached 90% of the maximum concentration), (3) the percentage of sugar consumed at which the production rate was maximal, (4) the percentage of volatile compound produced during the growth phase.

As it was not possible to calibrate the online GC system for the measurement of 2-phenylethanol and 2-phenylethylacetate, we represented these two molecules as the peak areas obtained in gas chromatography (Table 1). As both fermentations were performed at the same temperature, the raw data can be used to compare the performances of EC1118 and ECA5.

For the two strains, the plot of total production of each volatile compound, with the exception of propanol, against sugar consumption revealed that production yield was not constant throughout the fermentation process (Figures 1a to 1h and 2a to 2h). The fermentative aroma molecules were mostly produced during the stationary phase (Tables 2a and 2b) and the production yield was maximal during this phase (Figures 1a to 1h and 2a to 2h). Higher alcohols and esters of higher alcohols can be produced both from nitrogen (via the Erhlich pathway) and carbon metabolism (Swiegers *et al.*, 2005). As nitrogen (ammonium and amino acids) is exhausted during the stationary phase, this indicates that central carbon metabolism is the major contributor to the synthesis of these volatile compounds during wine fermentation.

3.1. Production of higher alcohols

3.1.1. Propanol

The pattern of production for propanol was different from that of the other volatile molecules studied. Propanol production by both yeast strains on both musts stopped at the end of the growth phase (Tables 2a and 2b). This is consistent with previous studies indicating that propanol is a metabolic marker of the availability of intracellular nitrogen (Mouret *et al.*, 2014b). This is, in turn, consistent with propanol being generated exclusively from nitrogen metabolism, by direct deamination of exogenous threonine or by interconversion between other amino acids and threonine, catalyzed by aminotransferases and transaminases (Boulton *et al.*, 1995). The overall production of this higher alcohol throughout the fermentation was similar for the two strains (Table 1). The maximum values of the production yield from sugar and of the production rate were also similar (Tables 3a and 3b). Therefore, this part of metabolism has not been significantly modified in the evolved strain ECA5.

3.1.2. 2-phenylethanol

In both culture media, 2-phenylethanol production by ECA5 was systematically greater than that by EC1118 (Table 1), and most notably during the growth phase. When the maximum cell count was reached, the peak area for 2-phenylethanol was 103% higher in natural must for ECA5 than for EC1118 and 97% higher in synthetic must whereas; however, at the end of the fermentation process, there was only 58% and 25% more in natural and synthetic musts, respectively (Tables 2a and 2b).

A plausible explanation for higher synthesis of 2-phenylethanol by ECA5 than EC1118 is the redirection of the carbon flux from glycolysis towards the pentose phosphate (PP) pathway in the evolved strain. Indeed, 2-phenylethanol is a

degradation product of phenylalanine, an amino acid produced from erythrose-4-phosphate, an intermediary of the PP pathway. Therefore, the level of 2-phenylethanol synthesis may be considered as a metabolic indicator of the flux through the PP pathway. The greater difference in the production of 2-phenylethanol between the two strains during the growth phase is consistent with the activity of the PP pathway being maximal during this phase. The PP pathway has indeed a major role in producing NADPH and precursors for biomass synthesis.

In parallel, an inverse relationship was observed between the final production of 2-phenylethanol and that of acetic acid (determined by the measurement of volatile acidity, Table 1). As already showed by ^{13}C flux analysis (Cadiere *et al.*, 2011), the reduced acetate excretion by ECA5 is mainly due more acetate being used for lipid biosynthesis, which is a NADPH consuming pathway.

3.1.3. Isobutanol and isoamyl alcohol

More isobutanol and isoamyl alcohol were produced by ECA5 than EC1118 in natural must (Table 1), as observed by Cadiere *et al.*, 2011, 2012. For each higher alcohol, the start and end of synthesis, the percentage production during the growth phase and the percentage of sugar consumed at which the production rate was maximal were all similar for the two strains (Table 2a). However, the maximum value of the rate of synthesis and the production yield during the stationary phase were higher for ECA5 (Table 3a). These findings indicate that the greater overall production of these two volatile molecules by ECA5 is not due to a modification of the chronology of synthesis but rather to higher conversion of α-keto acids into their corresponding higher alcohols (Figures 3a and 3b). Possibly, the activities or affinities of the enzymes involved in the production of isobutanol and isoamyl alcohol

from their α-keto acid precursors are higher in the evolved than parental strain. Also, the pools of α-keto acids and the distribution of their fluxes toward the synthesis of amino acids and the production of higher alcohols, may differ between the two strains.

In synthetic must, more isobutanol was produced by ECA5 whereas the production of isoamyl alcohol was comparable for the two strains (Table 1 and Figures 2b and 2c). Differences in the kinetic profiles were also noted. The production rate of isobutanol peaked at the same percentage of sugar consumed for the two strains (Table 2b). By contrast, for isoamyl alcohol, the percentage of sugar consumed at which the production rate was maximal was much lower for EC1118 than ECA5 (Table 2b). These findings indicate that environmental parameters affect the synthesis of these two higher alcohols by the two strains differently. One or several components of the synthetic medium may inhibit the synthesis of isoamyl alcohol by ECA5, without modifying that of isobutanol. One of the major differences between the natural and synthetic media is the lipid source: phytosterols in natural must and ergosterol in synthetic medium. To test whether the nature of the lipid source contributed to the regulation of the synthesis of these compounds, we used synthetic media containing either ergosterol or a mixture of phytosterols (β-sitosterol 90%, campesterol 5% and stigmasterol 5%) as lipid source: we determined the final concentrations of higher alcohols in cultures of the two strains in these media. Final concentrations of isobutanol were higher for ECA5 than EC1118 for the two lipid sources (Table 4). By contrast, isoamyl alcohol production by the evolved strain was higher than that of the parental strain only in the medium containing phytosterols; in the presence of ergosterol, the final contents of isoamyl alcohol were similar for the

two strains. These observations indicate that the production pathways of isobutanol and isoamyl alcohol are differently affected by the nature of the sterols available in the culture.

3.2. Production of esters

3.2.1. Acetates of higher alcohols

The four acetates of higher alcohols studied in this work (ethyl, isobutyl, isoamyl and phenyl acetates) were systematically produced in greater quantities by ECA5 than EC1118, as observed in other grape musts (Cadiere *et al.*, 2011, 2012). The overall production, the production yield during the stationary phase and the maximal value of the production rate were higher for the evolved strain than the parental strain in both fermentation conditions (Tables 1, 3a and 3b). Additionally, the difference in production of these esters between the two strains was larger than that of their corresponding higher alcohol precursors (Table 1). This suggests that the activity of the alcohol acetyl transferases (Atf1p and Atf2p) responsible for the bioconversion of higher alcohols into esters (Saerens *et al.*, 2010) might be higher in the evolved strain. Alternatively, the coordinated increased production of acetates of higher alcohols may have been due to a higher availability of acetyl-CoA.

For ethyl acetate and isoamyl acetate, differences in the level of synthesis between the strains were coupled to differences in the chronology of production. Indeed, although the timings of the start and end of synthesis were similar for the two strains (Tables 2a and 2b), the production profiles differed (Figures 4a to 4d): the rates of production of these two molecules peaked at different percentages of sugar consumed for the two strains (Tables 2a and 2b). These observations suggest

differences in the regulation of the synthesis of the acetates of higher alcohols between EC1118 and ECA5.

3.2.2. Ethyl esters

Greater production of ethyl hexanoate and ethyl octanoate by ECA5 was not systematic, but dependent on the fermentation medium (Table 1). A previous study (Cadiere *et al.*, 2012) comparing final production between EC1118 and ECA5 on four natural grape musts reported greater production by ECA5 on three musts whereas, the final concentrations were identical for the two strains in one must. This indicates that these ethyl esters cannot be used to differentiate between ECA5 and its parental strain in all fermentation conditions.

Although the overall production of ethyl esters was not necessarily different between strains EC1118 and ECA5 (Tables 1, 3a and 3b), the corresponding kinetic profiles were very different for the two strains on the two musts (Figures 5a to 5d). Indeed, the regulation of the synthesis of these molecules appeared to be very different between the two strains. Ethyl esters are produced through lipid metabolism, so differences in their regulation presumably reflect changes in the regulation of lipid synthesis in the evolved strain.

ECA5 produces four times as much lipid as EC1118 consistent with such a modification of lipid metabolism (Cadiere *et al.*, 2011). The different regulation of lipid metabolism in ECA5 may be the consequence of the management of the pool of redox cofactors. Indeed, the NADPH surplus generated by the greater flux through the PP pathway in ECA5 than EC1118 may be used for fatty acid synthesis which consumes large quantities of NADPH (Cadiere *et al.*, 2011).

4. Conclusion

In this work, we used an online monitoring system to compare the kinetic profiles of synthesis of various fermentative aroma compounds between the evolved strain ECA5 and its ancestral strain EC1118 in two culture media. This original tool allowed us to observe substantial differences in the regulation of the synthesis of several volatile compounds. One key finding was that 2-phenylethanol production was systematically higher by ECA5 than EC1118 and particularly during the growth phase. This finding is entirely consistent with the strategy used to obtain the evolved strain. Indeed, this higher alcohol is a degradation product of phenylalanine, an amino acid produced in the PP pathway that was the pathway targeted for increased flux by the adaptive evolution approach. Comparative analysis of the chronology of synthesis of aroma also revealed differences in the regulation of the synthesis of acetates of higher alcohols and ethyl esters, and systematically greater production of acetates of higher alcohols. The strain ECA5 has been reported to have reduced acetate production, as the result of an increase in carbon flux from acetate towards acetyl-CoA and lipid synthesis (Cadiere *et al.*, 2011). Here, we report findings highlighting substantial differences in the regulation of metabolism in ECA5 and the ancestral strain, which presumably result from extensive reshaping of the central metabolism. Adaptive evolution can lead to large-scale rewiring of gene regulatory networks (Dragosits and Mattanovich, 2013). Thus, in-depth analysis of the regulation of the synthesis of aroma compounds according to various fermentative parameters (temperature, and nitrogen, lipid or oxygen availability) may help elucidate the metabolic specificities of ECA5. Moreover, further insights into ECA5

metabolism might be obtained by combining gene expression analysis with the dynamic study of the synthesis of aroma compounds.

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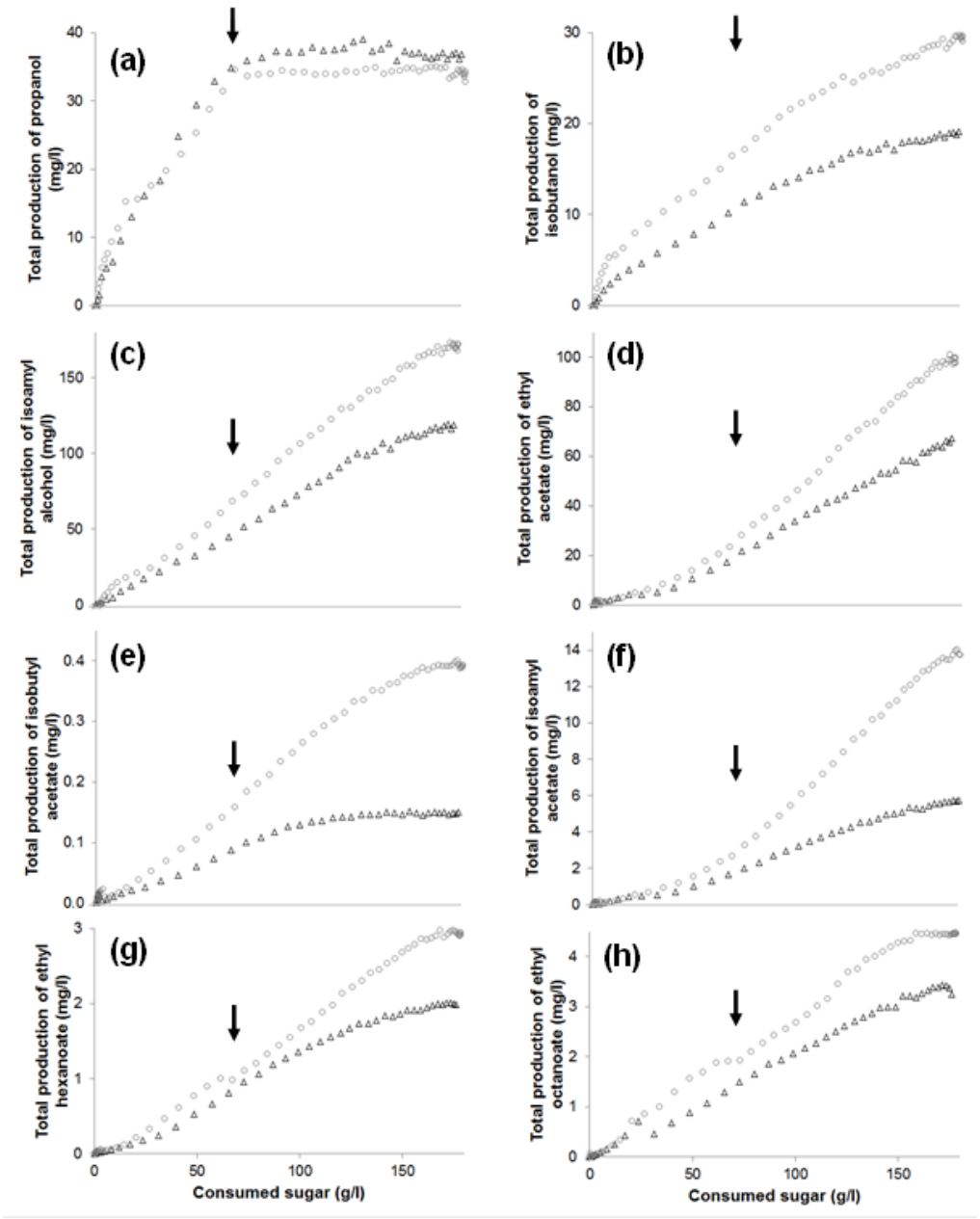

Fig. 1. Total production of propanol **(a)**, isobutanol **(b)**, isoamyl alcohol **(c)**, ethyl acetate **(d)**, isobutyl acetate **(e)**, isoamyl acetate **(f)**, ethyl hexanoate **(g)**, ethyl octanoate **(h)** by EC1118 (Δ) and ECA5 (o) in natural must as a function of sugar consumption. The growth phase ends when 67 g/L sugars are consumed, after 64h for EC1118 and 80h for ECA5 respectively and is indicated by an arrow.

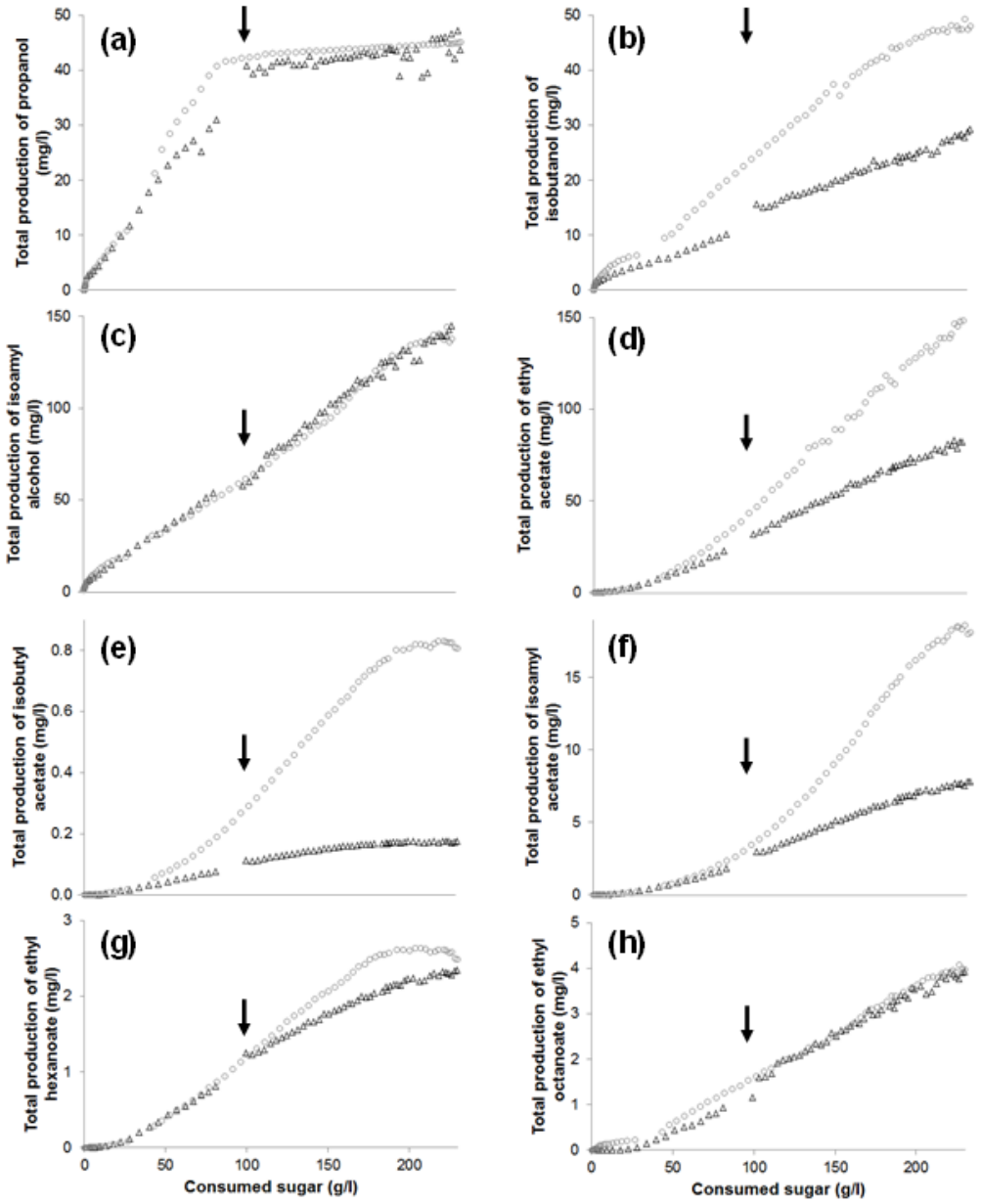

Fig. 2. Total production of propanol **(a)**, isobutanol **(b)**, isoamyl alcohol **(c)**, ethyl acetate **(d)**, isobutyl acetate **(e)**, isoamyl acetate **(f)**, ethyl hexanoate **(g)**, ethyl octanoate **(h)** by EC1118 (Δ) and ECA5 (o) in synthetic medium as a function of sugar consumption. The growth phase ends when 91 g/L sugars are consumed, after 70h for EC1118 and 100h for ECA5 respectively and is indicated by an arrow.

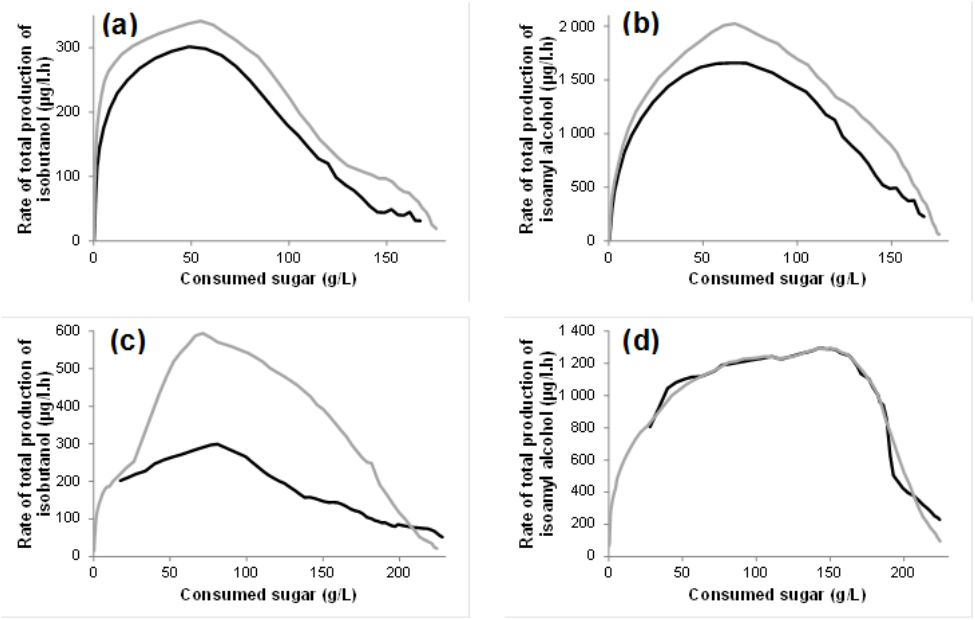

Fig. 3. Rate of total production of isobutanol **(a)** and isoamyl alcohol **(b)** in natural must. Rate of total production of isobutanol **(c)** and isoamyl alcohol **(d)** in synthetic medium. On each graph, data for EC1118 (black) and ECA5 (gray) are presented.

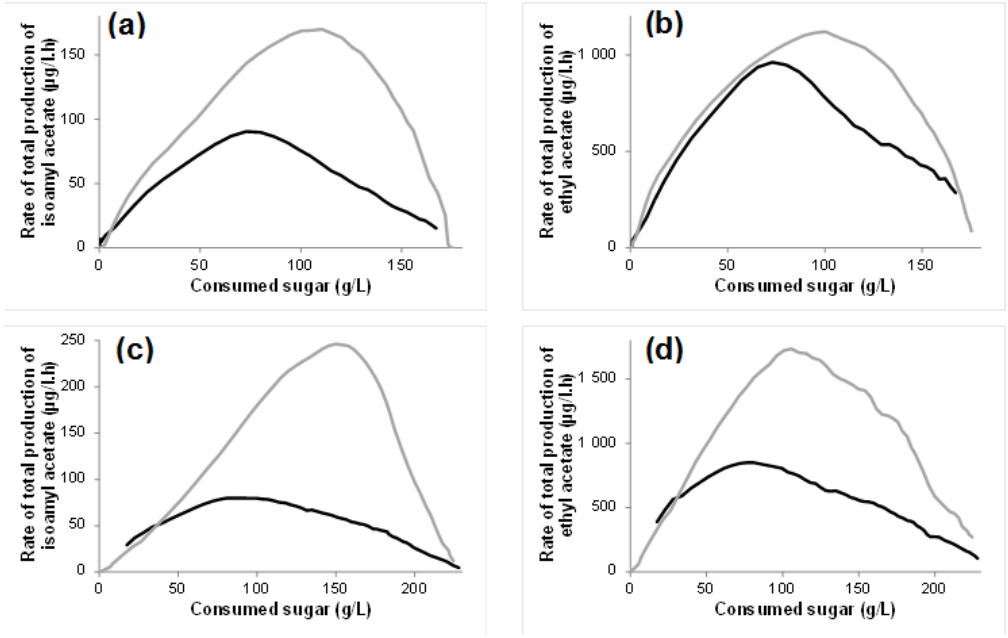

Fig. 4. Rate of total production of isoamyl acetate **(a)** and ethyl acetate **(b)** in natural must. Rate of total production of isoamyl acetate **(c)** and ethyl acetate **(d)** in synthetic medium. On each graph, data for EC1118 (black) and ECA5 (gray) are presented.

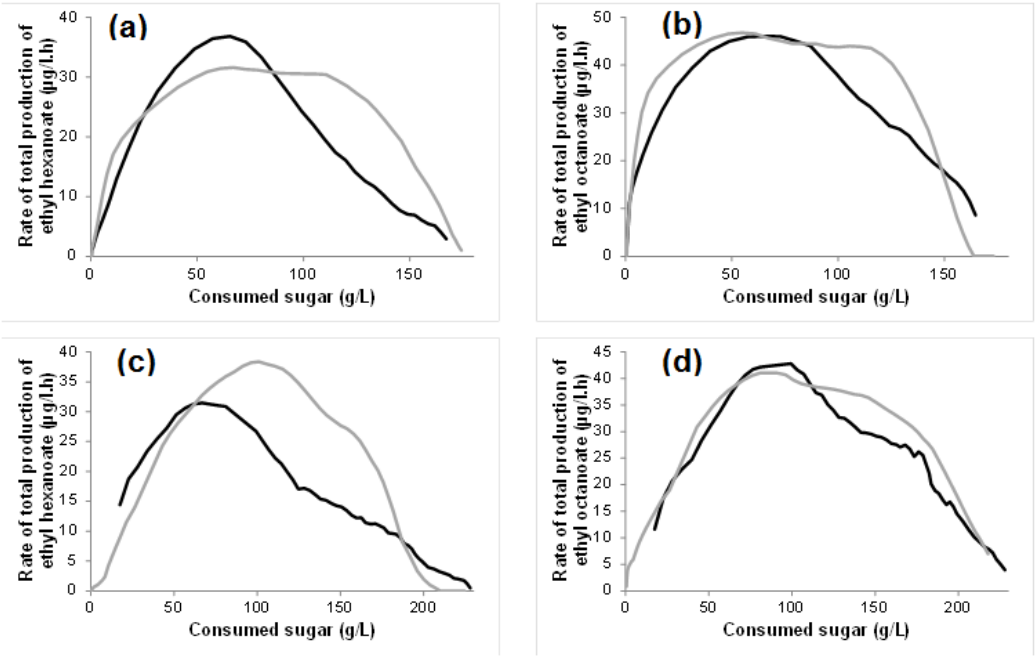

Fig. 5. Rate of total production of ethyl hexanoate **(a)** and ethyl octanoate **(b)** in natural must. Rate of total production of ethyl hexanoate **(c)** and ethyl octanoate **(d)** in synthetic medium. On each graph, data for EC1118 (black) and ECA5 (gray) are presented.

Table 1
Total production of volatile compounds

		Propanol (mg/l)	Isobutanol (mg/l)	Isoamyl alcohol (mg/l)	Isobutyl acetate (mg/l)	Isoamyl acetate (mg/l)	Ethyl acetate (mg/l)	Ethyl hexanoate (mg/l)	Ethyl octanoate (mg/l)	2-phenyl ethanol (mV)	2-phenyl ethyl acetate (mV)	Volatile acidity (g/l H_2SO_4)
Natural must	EC1118	39.0	19.1	119	0.168	6.66	79.4	2.14	3.43	6.37	4.91	0.28
	ECA5	34.7	29.8	174	0.401	14.06	101.4	2.99	4.71	10.07	12.24	0.16
	% difference	**-11%**	**56%**	**45%**	**138%**	**111%**	**28%**	**39%**	**37%**	**58%**	**149%**	**-43%**
Synthetic medium	EC1118	48.9	30.5	150	0.177	7.83	87.7	2.34	3.97	2.64	4.70	0.64
	ECA5	45.1	50.2	144	0.832	18.64	153.8	2.64	4.08	3.31	6.24	0.55
	% difference	**-8%**	**65%**	**-4%**	**371%**	**138%**	**75%**	**13%**	**3%**	**25%**	**33%**	**-14%**

% difference is reported as the percentage by which production by ECA5 is greater than that by EC1118.

Table 2
Kinetic parameters for the total production of higher alcohols and esters in natural (a) and synthetic (b) media

(a)

Natural must		Propanol	Isobutanol	Isoamyl alcohol	Isobutyl acetate	Isoamyl acetate	Ethyl acetate	Ethyl hexanoate	Ethyl octanoate	2-phenyl ethanol	2-phenyl ethylacetate
Start of synthesis*	EC1118	2%	5%	10%	28%	22%	28%	18%	10%	1%	1%
	ECA5	2%	2%	8%	15%	27%	23%	15%	11%	1%	1%
End of synthesis*	EC1118	41%	78%	84%	86%	97%	97%	91%	86%	80%	86%
	ECA5	38%	84%	84%	80%	90%	93%	84%	80%	71%	76%
Timing of the maximal production rate*	EC1118	18%	28%	37%	32%	41%	41%	37%	37%	-	-
	ECA5	19%	31%	38%	35%	62%	57%	38%	31%	-	-
% of volatile compound produced during the growth phase	EC1118	**90**	**53**	**38**	**52**	**25**	**22**	**38**	**38**	**42**	**49**
	ECA5	**99**	**55**	**40**	**40**	**20**	**24**	**33**	**43**	**54**	**41**

(b)

Synthetic medium		Propanol	Isobutanol	Isoamyl alcohol	Isobutyl acetate	Isoamyl acetate	Ethyl acetate	Ethyl hexanoate	Ethyl octanoate	2-phenyl ethanol	2-phenyl ethylacetate
Start of synthesis*	EC1118	5%	5%	7%	14%	22%	19%	17%	22%	14%	14%
	ECA5	4%	6%	6%	24%	32%	24%	18%	18%	2%	18%
End of synthesis*	EC1118	42%	91%	90%	69%	84%	93%	79%	84%	69%	91%
	ECA5	34%	83%	85%	77%	86%	91%	72%	86%	67%	78%
Timing of the maximal production rate*	EC1118	14%	34%	22%	28%	34%	34%	28%	42%	-	-
	ECA5	18%	30%	45%	49%	64%	45%	43%	34%	-	-
% of volatile compound produced during the growth phase	EC1118	**88**	**51**	**38**	**65**	**38**	**36**	**53**	**29**	**28**	**20**
	ECA5	**93**	**45**	**39**	**29**	**17**	**25**	**40**	**35**	**44**	**29**

* The start of synthesis, the end of synthesis, and the timing of the maximal production rate are expressed as percentages of sugar consumed.

Table 3
Production yields of fermentative aroma compounds from sugars during the stationary phase, maximum value of the production rate of the different volatile compounds for in natural (a) and synthetic (b) media

(a)

Natural must		Propanol	Isobutanol	Isoamyl alcohol	Isobutyl acetate	Isoamyl acetate	Ethyl acetate	Ethyl hexanoate	Ethyl octanoate
Production yield (mg/g)	EC1118	-	9.67e-2	7.12e-1	1.14e-3	1.02e-1	4.32e-1	1.30e-2	2.06e-2
	ECA5	-	1.34e-1	1.02	2.55e-3	3.75e-1	6.60e-1	1.79e-2	2.85e-2
	% difference	**-**	**39%**	**43%**	**124%**	**268%**	**53%**	**38%**	**38%**
Maximum rate (µg/l.h)	EC1118	1004	302	1659	3.31	90.3	962	36.8	46.1
	ECA5	988	341	2025	5.29	170	1120	31.6	46.8
	% of increase	**-2%**	**13%**	**22%**	**60%**	**88%**	**16%**	**-14%**	**2%**

(b)

Synthetic medium		Propanol	Isobutanol	Isoamyl alcohol	Isobutyl acetate	Isoamyl acetate	Ethyl acetate	Ethyl hexanoate	Ethyl octanoate
Production yield (mg/g)	EC1118	-	1.22e-1	6.17e-1	7.65e-4	3.85e-2	4.00e-1	1.33e-2	1.93e-2
	ECA5	-	2.21e-1	6.12e-1	5.14e-3	1.13e-1	7.68e-1	1.66e-2	1.87e-2
	% difference	**-**	**81%**	**-1%**	**572%**	**194%**	**92%**	**25%**	**-3%**
Maximum rate (µg/l.h)	EC1118	1012	300	1272	2.61	79.9	851	31.5	42.8
	ECA5	1083	594	1243	12.3	246	1735	38.4	41.1
	% of increase	**7%**	**99%**	**-2%**	**371%**	**208%**	**104%**	**22%**	**-4%**

Table 4
Final concentration of higher alcohols in synthetic musts (in the liquid phase) containing either 3.75 mg/l ergosterol or phytosterols as the lipid source

		Isobutanol (mg/l)	Isoamyl alcohol (mg/l)
Ergosterol	EC1118	55.2	159
	ECA5	78.4	165
	% difference	**42%**	**3%**
Phytosterols	EC1118	40.5	105
	ECA5	60.3	134
	% difference	**49%**	**27%**