Food Microbiology 33 (2013) 228–234

Contents lists available at SciVerse ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Genetic analysis of geraniol metabolism during fermentation

Damien Steyer [a,b,1], Claude Erny [c], Patricia Claudel [a], Geneviève Riveill [a], Francis Karst [a,b], Jean-Luc Legras [a,b,d,e,f,*]

[a] INRA, UMR1131, F-68021 Colmar, France
[b] Université de Strasbourg, UMR1131, 28 rue de Herrlisheim, BP 568, 68 008 Colmar, France
[c] Université de Haute Alsace, EA3991 Laboratoire Vigne Biotechnologies et Environnement, 29 rue de Herrlisheim, BP 568, 68 008 Colmar, France
[d] INRA, UMR1083, Sciences pour l'Oenologie, F-34060 Montpellier, France
[e] SupAgro, UMR1083, Sciences pour l'Oenologie, F-34060 Montpellier, France
[f] Université Montpellier I, UMR1083, Sciences pour l'Oenologie, F-34060 Montpellier, France

ARTICLE INFO

Article history:
Received 6 May 2012
Received in revised form
16 July 2012
Accepted 14 September 2012
Available online 11 October 2012

Keywords:
Saccharomyces cerevisiae
Fermentation
Geraniol
Geranyl acetate
Citronellol
OYE2
ATF1

ABSTRACT

Geraniol produced by grape is the main precursor of terpenols which play a key role in the floral aroma of white wines. We investigated the fate of geraniol during wine fermentation by *Saccharomyces cerevisiae*. The volatile compounds produced during fermentation of a medium enriched with geraniol were extracted by Stir-bar sorptive extraction and analysed by GC–MS. We were able to detect and quantify geranyl acetate but also citronellyl- and neryl-acetate. The presence of these compounds partly explains the disparition of geraniol. The amounts of terpenyl esters are strain dependant. We demonstrated both by gene overexpression and gene-deletion the involvement of *ATF1* enzyme but not *ATF2* in the acetylation of terpenols. The affinity of *ATF1* enzyme for several terpenols and for isoamyl alcohol was compared. We also demonstrated that *OYE2* is the enzyme involved in geraniol to citronellol reduction. Fermenting strain deleted from *OYE2* gene produces far less citronellol than wild type strain. Moreover lab strain over-expressing *OYE2* allows 87% geraniol to citronellol reduction in bioconversion experiment compared to about 50% conversion with control strain.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Consumer interest towards alcoholic beverages produced by saccharomyces such as wine and beer is mainly linked to their complex aroma. Many chemical components are involved in these aromas, including volatile acetate esters and terpenoids (Francis and Newton, 2005). Acetate esters such as isoamylacetate and phenylethyl acetate respectively confer banana and flowery aroma to the wines or beer. Although well known, the involvement of acetate esters in wine aroma is difficult to fully measure because of their additive effect (van der Merwe and van Wyk, 1981; Francis and Newton, 2005). The synthesis of these esters and of a broad range of other acetate esters is under the control of *ATF1* gene (Verstrepen et al., 2003).

Another chemical family involved in aromatic profile of fermented beverages is the terpenols family. Monoterpenoids are 10-carbon compounds with strong sensory properties. They are widely found in plants and are the main constituents of essential oils. Terpenols are found in beer (Takoi et al., 2010) and linalool has been described as an important contributor of the floral aroma of hops (Stucky and McDaniel, 1997). In Wines, terpenols are key components or aromatic profiles (Schreier and Jennings, 1979) and linalool, geraniol, nerol, citronellol and α-terpineol are found in several wines with "floral" character, such as muscat (Ribereau-Gayon et al., 1975), gewürztraminer (Guth, 1997), riesling (Simpson and Miller, 1983) and chardonnay wines (Duchêne et al., 2009). In muscat and gewürztraminer wines the total content of linalool or geraniol can reach up to 1.5 and 4 mg/L respectively, in contrast with the other wines cited where the content are 10–20 fold lower.

Terpenoids content of wines result from the combined effects of must composition, fermenting yeasts metabolism and chemical evolution. In the grape vine berries monoterpenoids are synthesized from geranyl diphosphate through the DOXP/MEP pathway (Luan and Wüst, 2002) and they can be found both free and

* Corresponding author. INRA, UMR1083, Montpellier SupAgro, 2 place Pierre Viala, 34 060 Montpellier, France. Tel.: +33 4 99 61 31 70; fax: +33 4 99 61 28 57.
E-mail address: jean-luc.legras@supagro.inra.fr (J.-L. Legras).
[1] Present address: Twistaroma, F-68021 Colmar, France.

0740-0020/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fm.2012.09.021

glycosidically-bound. Geraniol is the main precursor of monoterpenoids in the must. During fermentation the primary monoterpenic must composition is modified by wine yeasts such as *Saccharomyces cerevisiae*, *Saccharomyces uvarum* and hybrids (Gamero et al., 2011; King and Dickinson, 2003). These yeasts have only a very limited capability to produce monoterpenoids, limited to a few µg/L amounts (Carrau et al., 2005), but they can release bound terpenoids thanks to their glycosidase activity (Fernández-González et al., 2003; Ugliano et al., 2006; Zoecklein et al., 1997) or modify the free terpenoids content (Fia et al., 2005; King and Dickinson, 2000). Adding known amounts of geraniol to must at the beginning of fermentation has revealed that a drastic reduction of monoterpenols level occurs at the beginning of fermentation (Vaudano et al., 2004). This drastic reduction is partly explained by documented reactions including the transformation of geraniol to linalool and nerol by acid-catalysis or its reduction to citronellol by unknown yeast enzymes (Gamero et al., 2011; Gramatica et al., 1982; King and Dickinson, 2000). However the total level of monoterpenols produced and residual geraniol hardly reached 40% of the initial geraniol amount (Vaudano et al., 2004). This loss of geraniol has been attributed to evaporation, incorporation in yeast membranes, acetylation (King and Dickinson, 2003) or metabolization into ergosterol (Vaudano et al., 2004). Indeed, in animals and plants farnesol might be recycled into the farnesyl diphosphate precursor of sterols (Bentinger et al., 1998; Fitzpatrick et al., 2011). Nevertheless neither farnesol nor geraniol has been reported to support growth of *S. cerevisiae* sterol auxotrophic mutants. Finally the free terpenoids profile can be modified chemically, especially while wine ageing due to the acidic pH of wines (Simpson and Miller, 1983).

In this work we have tried to identify the yeast enzymes involved in the metabolization of monoterpenoids during fermentation. Geraniol metabolization during yeast growth was analysed either with geraniol exogenously provided or synthesized *in vivo* in engineered yeast strains. Our results present a new global view of the fate of geraniol metabolism during fermentation.

2. Materials and methods

2.1. Chemicals and reagents

Absolute ethanol was obtained from VWR. NaCl 99% was purchased from Sigma. Acetonitrile, isoamylacetate, 2-methyl butanol, 2-phenylethyl acetate, linalool, α-terpineol, citronellol, nerol, geraniol, linalylacetate, were supplied by Fluka.

2.2. Yeast strains

S. cerevisiae strains used were S288c and the oenological strain 59a, a haploid derivative from the sequenced EC1118 wine yeast strain (Novo et al., 2009). Strains BY4741 deleted for a single gene, i.e. ATF1, ATF2, OYE2 and OYE3 were from Euroscarf collection.

Yeast strain overexpressing *ATF1* was constructed by direct recombination in yeast. Yeast strain Fya 1679-28c was co-transformed with Not1-digested-pMO1 plasmid and *ATF1* ORF obtained by PCR with primers ATF1PMO1FW (CAAAAGAAA GAAAAAAAATATACCCCAGC**ATGAATGAAATCGATGAGAAAAATCAG**) and ATF1PMO1RV (GCAGCCCGGGGGATCCACTAGTT**CTAAGGGCCTA AAAGGAGAGC**). Fya 1679-28c bearing empty pMO1 plasmid was used as control.

2.3. Culture conditions

Fermentations were performed either in YPD medium containing 8% glucose (W/V) or in synthetic MS300 medium containing 20% glucose (W/V) which approximates to a natural must as previously described (Bely et al., 1990), with the following modification; in anaerobic factors ergosterol 15 mg/L was replaced by sitosterol (main sterol present in grape) 7 mg/L final concentration. All fermentations were led at 20 °C.

2.4. Bioconversion experiments

Cells were pre-grown in supplemented YNB medium, then in 200 ml YPD medium overnight at 28°. Cells were recovered in exponential growth phase, washed twice with ice cold water then with 50 mM phosphate buffer pH 7. Highly dense suspension of resting cells (DO600 nm = 20) were prepared in 50 ml phosphate buffer containing 2% glucose and complemented with 200 µg/ml of the compound tested as a bioconversion substrate. Medium was agitated at 28°.

2.5. Volatile compounds extraction

Volatile compounds were extracted from fermentation medium by Stir Bar Sorptive Extraction-Liquid Desorption (SBSE-LD). A stir bar (0.5 mm thick; 10 mm long) coated with a polydimethylsiloxane film (Twister; Gerstel, Müllheima/dRuhr; Germany) was immersed in 20 ml of fermentation supernatant, and stirred for 180 min at 1250 rpm at room temperature. For back-extraction purposes, the stir bars were rinsed with deionized water and gently dried then placed into a 250 µL glass flat-bottom inserts filled with 100 µL of acetonitrile inside a glass vial. The back-extraction was performed by ultrasonic treatment for 30 min.

Volatile compounds were extracted from bioconversion medium by liquid–liquid extraction with 1 ml pentane. Pentane solution was directly analysed by GC/MS.

2.6. Volatile compounds analysis

Samples were analysed by GC–MS using an Agilent 6890N gas chromatograph equipped with an Agilent 7683 automatic liquid sampler coupled to an Agilent 5975B inert MSD (Agilent Technologies).

The gas chromatograph was fitted with a DB-Wax capillary column (60 m × 0.32 mm i.d. × 0.50 µm film thickness, J&W Scientific) and helium was used as carrier gas (1 ml min-1 constant flow).

The GC oven temperature was programmed without initial hold time at a rate of 2.7 °C min-1 from 70 °C to 235 °C (hold 10 min). The injector was set to 250 °C and used in pulsed splitless mode (25 psi for 0.50 min). The temperatures of the interface, MS ion source and quadrupole were 270 °C, 230 °C and 150 °C, respectively.

The Mass spectrometer was operated in electron impact ionization mode (EI, 70 eV) and the masses were scanned over an *m*/*z* range of 29–300 amu.

Agilent MSD ChemStation software (G1701DA, Rev D.03.00) was used for instrument control and data processing. The mass spectra were compared with the Wiley's library reference spectral bank.

In synthetic wine, quantification of geraniol, citronellol, linalool, α-terpineol and nerol was based on calibration while terpenyl acetates quantification was based on linalylacetate calibration curve.

3. Results

3.1. Formation of terpenyl acetates partly explains geraniol disappearance

The loss of volatiles caused by CO_2 stripping during alcoholic fermentation can be significant with 20% and 40% for isoamylacetate and ethyl esters respectively (Morakul et al., 2012). To

analyse the fate of geraniol during fermentation, we first investigated the loss potentially caused by CO_2 stripping during fermentation. We therefore used Erlenmeyer flasks containing 6% ethanolic water solutions supplemented with geraniol 20 or 40 mg/L final concentrations. Air was flushed through the flasks and stopped with a C18-SPE cartridge. After 64 h at 28° with agitation, volatiles were extracted from medium and SPE cartridge with dichloromethane. The results showed that at 20 and 40 mg/L geraniol, about 22% of the terpenoids were retained on the cartridge. These figures were slightly higher than those obtained by Ferreira et al. (1996) for octanol and linalool (9.5 and 7.5% at 27 °C respectively) for lower terpenol concentrations (1 mg/L) and can be seen as an upper limit. In addition, these authors also estimated that the loss was lower than 1% at 17 °C indicating that the loss is moderate when fermenting at 20 °C.

We next undertook fermentation process with two different yeast strains, namely the laboratory strain S288c and a haploid strain 59a derived from wine strain EC1118 (Novo et al., 2009). These strains are unable to produce geraniol. To mimic the terpenic must composition the synthetic fermentation medium (MS300) was supplemented with geraniol at 1 mg/L. Volatile compounds were extracted at various times from the fermentation medium by SBSE and analysed by GC–MS. The results, Fig. 1, illustrate that for both strains, geraniol rapidly disappeared from the medium early in the fermentation process (Fig. 1, panel A) while citronellol, linalool and nerol appeared in the medium after a few hours (Fig. 1, Panel B), citronellol being the main component produced. No remarkable differences were observed between laboratory and oenological strains concerning the terpenols levels. After about 25 h of fermentation, geranyl- and citronellyl acetate were detected (Fig. 1, panel C). The concentrations of these compounds were strain dependent. The oenological 59a strain exhibited the strongest ability to produce acetates. Nevertheless it is noteworthy that whatever the strain, the amount of terpenyl-acetate decreased after 50 h (59a strain) or 100 h (S288c strain) suggesting a lack of stability in the medium.

Quantification of geraniol-derived terpenols allowed us to express the amounts of terpenols and acetates as a percentage of added geraniol (Fig. 2). Our analysis revealed that 50% (S288c strain) and up to 80% (59a strain) of the geraniol added at the beginning of fermentation were recovered as terpenols or terpenyl acetates. Presence of terpenyl acetates in fermentation media has already been reported (King and Dickinson, 2003), but this work is the first evidence of the importance of terpenyl acetates as a fate for must geraniol.

Even when taking into account the presence of terpenyl acetates, about 50% or 20% of initial geraniol amount is lost at the end of fermentation, depending on yeast strain. It should be mentioned that additional monoterpenes were identified, such as myrcene and pinene for example. As we did not have established calibration curves for these compounds they were therefore not quantified.

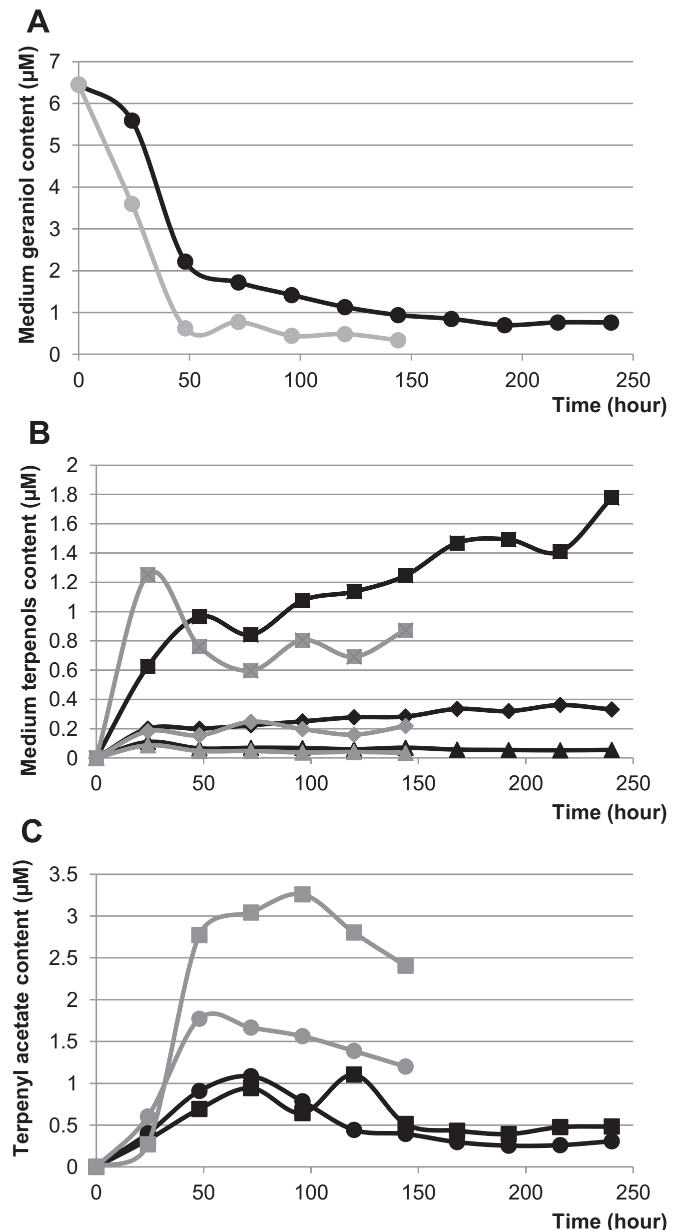

Fig. 1. Time course of terpenols and terpenyl acetates in fermentation medium. Geraniol (panel A), terpenols (panel B) and terpenyl acetates (panel C) were quantified along fermentation of MS300 medium + 1 mg/L geraniol with S288c lab strain (black line) or 59a oenological strain (grey line). Quantified terpenols included citronellol (squares), linalool (diamonds) and nerol (triangles). Terpenyl acetates included citronellyl-acetate (squares) and geranyl-acetate (circles).

3.2. *ATF1 alcohol acetyltransferase is the main contributor to terpenyl acetate synthesis*

Isoamyl-acetate and phenyl-ethyl acetate, as well as other acetates are known to be synthesized mainly by Atf1p (Verstrepen et al., 2003). We therefore investigated the role of Atf1p and the isoform Atf2p in the synthesis of terpenyl acetates.

The BY4741 strains deleted for *ATF1* or *ATF2* genes were fermented in YPG 8% medium complemented with 5 mg/L geraniol. YPG was chosen rather than synthetic must in order to favour rapid fermentations and minimize terpenyl acetate degradation. Geraniol amount was increased up to 5 mg/L to ease the analysis of geraniol derived products. The concentrations of terpenols and terpenyl acetates measured at the end of fermentation (Table 1) indicate that *ATF1* deletion drastically reduced the amounts of terpenyl acetates (66–88% reduction depending to the terpenyl acetate) while *ATF2* deletion had a lower impact (up to 31% reduction). The amounts of terpenols recovered were not significantly affected by *ATF1* deletion except for citronellol.

Involvement of *ATF1* in terpenyl acetate production was further evidenced by geraniol bioconversion experiments. Fy1679 strain transformed with empty pMO1 plasmid exhibited a basal level of geraniol acetylation corresponding to the presence of *ATF1* genomic copy, while *ATF1* over-expressing strain was more efficient in geraniol acetylation. Geranyl acetate seemed to reach equilibrium after 100 h (Fig. 3).

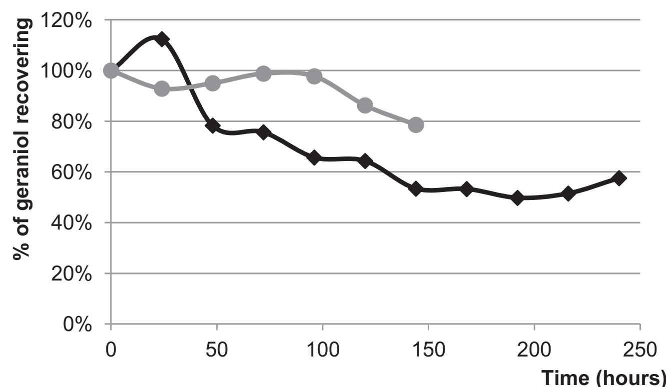

Fig. 2. Time course of total amounts of terpenyl acetates + terpenols in fermentation medium. Terpenols and terpenyl acetates were extracted by SBSE-LD from MS300 + 1 mg/L geraniol level fermented by S288C strain (black line) or 59a strain (grey line). Total amount of terpenoids is expressed as a molar percentage of added geraniol.

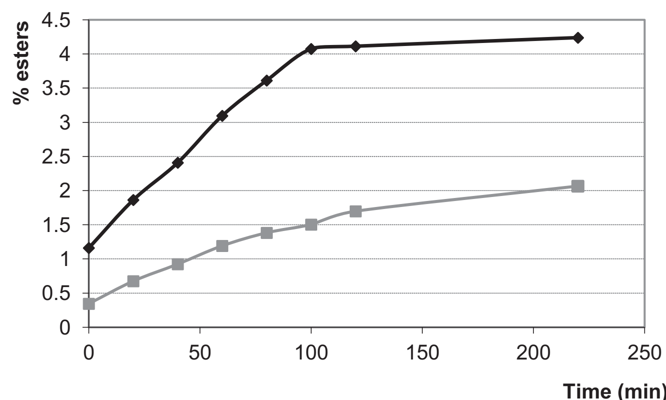

Fig. 3. Variations of terpenol acetylation obtained during a bioconversion experiment with lab strain transformed with empty pMO1 plasmid (grey line) or with pMO1 plasmid overexpressing *ATF1* (black line). Esters are expressed as a percentage of recovered terpenoids.

To have a better knowledge concerning Atf11p substrate specificity, we tested Atf1p activity on different alcohols in bioconversion experiments with the *ATF1* over-expressing strain. Fig. 4 shows that, as previously described (Verstrepen et al., 2003), isoamyl-alcohol is esterified by Atf1p. Moreover, Atf1p is able to esterify all the terpenols tested more efficiently than isoamyl alcohol. Nevertheless acetylation time courses vary with the substrates and citronellol acetylation seems to happen at the beginning of the bioconversion and then stabilize while geraniol and nerol acetylation could be observed during all the experiment. Geraniol and nerol acetylation was more experiment-dependent than isoamyl- or citronellol-acetylation.

Taken together, our results provide strong evidence that acetylation is one of the major processes of terpenols disappearance from musts during fermentation and that the main enzyme involved in this reaction is the alcohol acetyltransferase encoded by *ATF1*. Finally, the differences of acetylation observed between S288c and 59a strains during fermentation (Fig. 1 panel C) could not be explained by differences in *ATF1* coding sequences (results not shown).

3.3. *OYE2 is involved in geraniol to citronellol reduction during fermentation*

S. cerevisiae is known to be able to convert geraniol into R-citronellol (Gramatica et al., 1982) but the enzyme responsible for this transformation has not been identified.

Table 1
Concentration of geraniol derivatives produced by BY4741Δ*ATF1* and BY4741Δ*ATF2* after 48 h of fermentation on 8% glucose-YPG medium complemented with 5 mg/L geraniol.

	Conc in μg/L				
	BY4741 (*atf1*Δ)		BY4741 (*atf2*Δ)		BY4741 wt
Citronellol	**1837.68**	(**1.47**)	**1162.16**	(**0.93**)	1250.78
Geraniol	3152.09	(1.08)	2748.41	(0.94)	2925.63
Nerol	205.59	(1.15)	169.30	(0.95)	178.54
Linalool	25.33	(0.78)	30.54	(0.94)	32.43
∑ terpenol	5220.69	(1.19)	4110.41	(0.94)	4387.38
Citronellylacetate	**32.25**	(**0.12**)	229.48	(0.86)	268.12
Geranylacetate	**376.43**	(**0.33**)	**795.54**	(**0.69**)	1147.83
Nerylacetate	**6.5**	(**0.16**)	36.27	(0.88)	41.01
∑ terpenyl acetates	**415.19**	(**0.28**)	**1061.29**	(**0.73**)	1456.95
Isoamylalcohol	1263.91	(0.87)	1170.61	(0.80)	1459.98
Isoamylacetate	131.6	(0.13)	512.02	(0.52)	980.07

Volatile compounds were extracted by SBSE-LD and quantified by GC–MS. Results are the averages for three independent fermentations. Values in parentheses show the value relative to that of the wild type. Values that are significantly different from those of the wild-type strain ($\alpha = 0.05$) are in bold.

In literature it was shown that *OYE2* is able to reduce 3-methyl 2-buten-1-al to 3-methyl-2-butanal (Chou and Keasling, 2009). Considering the structural homologies between these two compounds and geranial and citronellal respectively, we hypothesized that transformation of geraniol to citronellol included aldehyde intermediates and that Oye2p was involved in geranial to citronellal reduction. Recently, Yuan et al. (2011) have shown that crude extracts of *Escherichia coli* cells expressing Oye2p are able to reduce geraniol into citronellol but *in vivo* activity has not been evidenced. Therefore we tested Oye2p and the homologous Oye3p for their possible involvement in geraniol to citronellol transformation in fermentation experiments.

BY4741 strains deleted for *OYE2* or *OYE3* were fermented in YPG 8% glucose containing 5 mg/L geraniol. Volatile compounds were analysed at the end of fermentations (Fig. 5). The deletion of *OYE2* resulted in a dramatic decrease of citronellol amount in fermentation medium and in an increase in linalool, nerol and geraniol concentrations. In contrast the deletion of *OYE3* gene had no impact on terpenols concentrations.

We next compared the effect of *OYE2* overexpression on geraniol reduction into citronellol in bioconversion experiments. Strain Fy (a) was transformed with empty pMO1 vector or with pMO1 vector

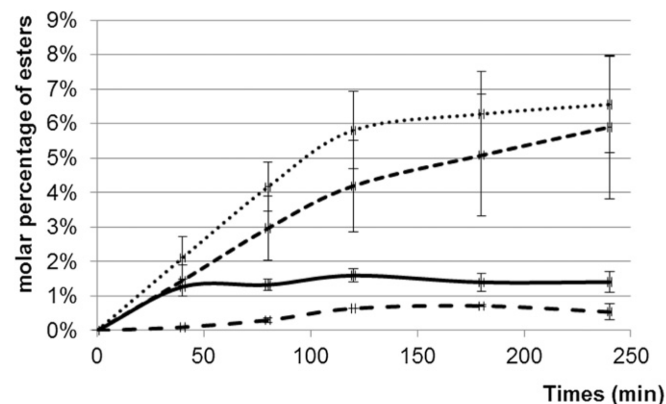

Fig. 4. Time course of acetylation of various terpenols obtained during bioconversion experiments with lab strain overexpressing *ATF1*. Acetate levels are expressed as molar percentages of the sum alcohol + acetate. Isoamyl acetate (▬ ▬), citronellyl acetate (▬▬), geranyl acetate (▬▬▬▬) and neryl acetate (▪▪▪▪▪) are extracted from cell-free supernatant after bioconversion of the corresponding substrate and measured by GC–MS. Results are the averages of two independent experiments. Standard errors are represented.

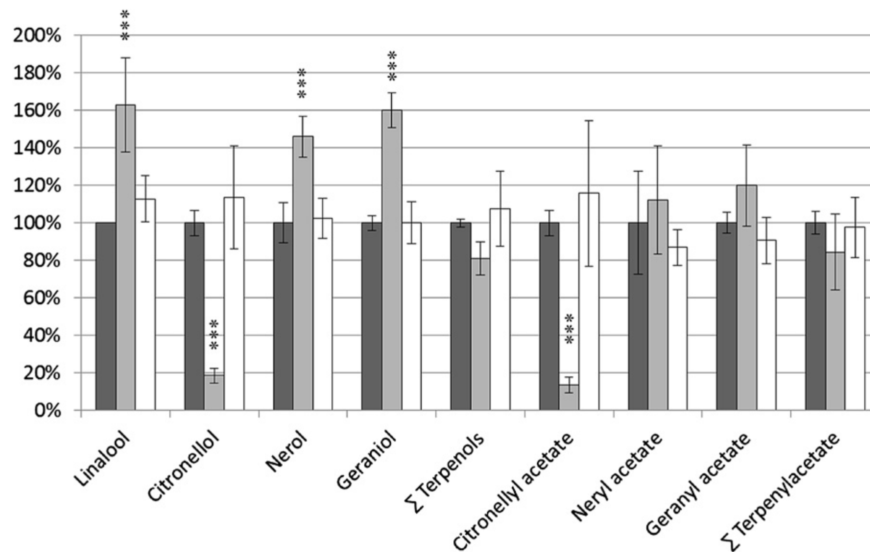

Fig. 5. Terpenols amounts produced after 48 h of fermentation in YPG 8% by BY4741ΔOYE2 (light grey) and BY4741ΔOYE3 (white) relative to those produced by – wild-type BY4741 strain (dark grey). Results are the averages of three independent fermentations. *** Values significantly ($\alpha = 0.05$) different from the wild-type strain.

carrying *OYE2* gene under the control of the strong *PMA1* promoter. Resting cells were suspended in 50 mM phosphate buffer pH 6.9 supplemented with 2% glucose and 200 μg/ml geraniol. The results, Fig. 6, show that for the control strain expressing the chromosomal copy of OYE2 the levels of citronellol and geraniol tend to an equimolar ratio after 70 h of incubation. In contrast, about 87% geraniol is reduced to citronellol for the strain overexpressing *OYE2* gene.

These results clearly establish that Oye2p is involved in the reduction of geraniol to citronellol during fermentation.

4. Discussion

Monoterpenols are the major components of the organoleptic properties of aromatic wines such as muscat or gewürztraminer, especially geraniol which enriches the wine with floral flavours. Using the sensitive SBSE method for volatiles analysis we investigated the fate of geraniol during fermentation and the role of fermenting yeasts in the final aromatic composition of wine. Our results show that, beneath terpenol conversion, the high loss of geraniol observed during fermentation is linked to esterification of geraniol itself and the derived terpenols. The enzyme activity involved in the esterification process is the alcohol acetyltransferase Atf1p. Several studies have revealed that among the three alcohol acetyltransferase genes in yeast genome, *ATF1*, *ATF2* and *lg-ATF1*, Atf1p is the main contributor of ethyl acetate and isoamyl acetate formation (Mason and Dufour, 2000; Verstrepen et al., 2003). Here we establish that terpenic alcohols are also good substrates for Atf1p.

Although esters represent the largest group of flavour compounds in alcoholic beverages, the importance of terpenyl acetates in alcoholic beverages aroma has not been investigated, maybe due to the lack of quantification method. According to literature terpenyl esters bring pleasant odour impression described as fruity, sweet floral for geranyl acetate (HSDB) and fresh, citrus for citronellyl acetate (Yamamoto et al., 2004). But the odour thresholds of these terpenyl acetates reach 250 ppm (Yamamoto et al., 2004) and 9–460 ppm (HSDB) for citronellyl acetate and geranyl acetate respectively while the corresponding terpenols can be perceived at 50 ppm (Yamamoto et al., 2004) and 30 ppm (Guth, 1997) respectively. Implication of terpenyl acetates themselves in wine aroma will be dependent of their concentrations and of possible interactions with other esters. Moreover, the presence of these terpenyl acetates is linked to lowered amounts of the corresponding terpenols and most probably to the diminution of the floral character of wines such as muscat or gewürztraminer. Therefore esterification of terpenic alcohols is not favourable in these wines. On the other hand, acetylation of geraniol prevents its reduction into citronellol, as can be observed with the deletion of *ATF1* which leads to a 50% increase of the production of citronellol. The poor stability of terpenyl acetates should allow further liberation of geraniol, so esterification could also be considered as a way to preserve the geraniol potential of wines.

The reduction of geraniol to citronellol may also change the floral character of the wines to citrus like character. We show here that Oye2p is the main enzyme involved in geraniol reduction. As suggested earlier (Gramatica et al., 1982) geranial is the precursor of R-citronellol produced by yeasts; in grape vine berries some geraniol is metabolized to S-citronellol (Luan and Wüst, 2002). Overexpression of *OYE2* directs in a bioconversion experiment an 84% conversion of geraniol to citronellol revealing that the enzymes involved first in the oxidation of geraniol to geranial, then in the second reduction of citronellal to citronellol are not limiting in the process. These oxido-reductases are yet unknown.

Oye2p and Oye3p are the two isoenzymes of Old Yellow Enzyme, the first discovered flavoprotein isolated from brewer's bottom yeast by Warburg and Christian, 1933. Oye2p is an NADPH oxidoreductase that contains flavin mononucleotide (FMN) as the prosthetic group. It has been shown that a large number of α, β-unsaturated aldehydes and ketones are able to act as electron acceptors (Vaz et al., 1995). For examples one can cite as best substrates, 2-cyclohexanone, N-ethyl maleimide, methyl vinyl ketone, 2-methyl and 2-ethyl acrolein and cinnamaldehyde. Oye2p catalyses the reduction of the double bond rather than the carboxyl group. We have not precisely determined the turnover number for geranial, however *in vitro* tests based on NADPH oxidation showed that the rate of geranial reduction is similar to that of cinnamaldehyde, suggesting that geranial could be one of the best substrates of Oye2p. The physiological role of Old Yellow Enzymes in yeast may be detoxification of unsaturated metabolites and reactive oxygen species (Odat et al., 2007; Trotter et al., 2006). Geraniol is known to be cytotoxic and to have antibacterial properties. One can hypothesize that the strong resistance to geraniol of yeast (>250 mg/L; Oswald et al., 2007) could be linked to its metabolization by Oye2p.

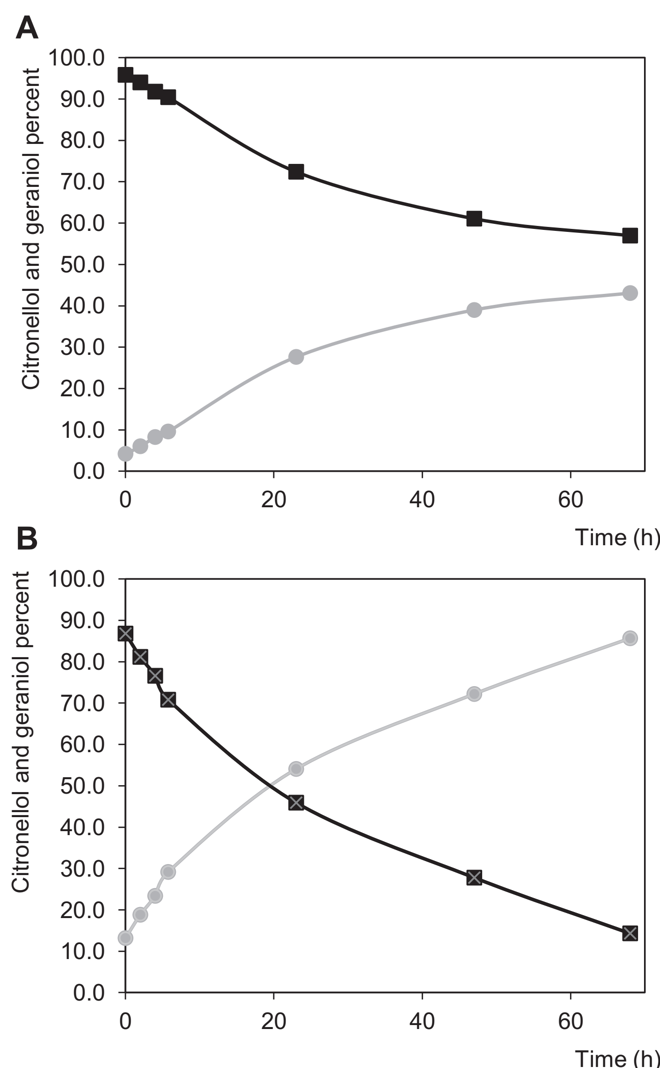

Fig. 6. Levels of citronellol (grey line) and geraniol (black line) obtained in bioconversion experiment with lab strain transformed with empty pMO1 plasmid (panel A) or with pMO1 plasmid overexpressing OYE2 (panel B). Citronellol and geraniol are expressed as a percentage of the sum of recovered citronellol and geraniol.

In conclusion, our study shows that the high geraniol disappearance during early fermentation is mainly linked to isomerization by acid-catalysis, reduction to citronellol by Oye2p and esterification by Atf1p. During fermentation, citronellol appears in the medium before terpenyl-acetates, consistent with oxygen repression of *ATF1* (Mason and Dufour, 2000). Nevertheless Atf1p and Oye2p enzymes compete to transform geraniol and these two genes could be good targets to select or engineer wine yeasts.

We could not identify the fate of 25% of the initial geraniol. Literature data of Ferreira et al. (1996) suggest that only few per cents of the initial geraniol pool may be stripped into the atmosphere by the carbon dioxide produced during fermentation. However one can also suppose that part of the unexplained loss may result from acid catalysis which produces new volatile terpenes that we have identified but not yet quantified.

References

Bely, M., Sablayrolles, J., Barre, P., 1990. Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in oenological conditions. Journal of Fermentation and Bioengineering 70 (4), 246–252.

Bentinger, M., Grunler, J., Peterson, E., Swiezewska, E., Dallner, G., 1998. Phosphorylation of farnesol in rat liver microsomes: properties of farnesol kinase and farnesyl phosphate kinase. Archives of Biochemistry and Biophysics 353, 191–198.

Chou, H.H., Keasling, J.D., 2009. Host Cells and Methods for Producing 3-Methyl-2-buten-1-ol, 3-Methyl-3-buten-1-ol, and 3-Methyl-butan-1-ol. WO Patent WO/2009/006,429.

Carrau, F.M., Medina, K., Boido, E., Farina, L., Gaggero, C., Dellacassa, E., Versini, G., Henschke, P.A., 2005. De novo synthesis of monoterpenes by *Saccharomyces cerevisiae* wine yeasts. FEMS Microbiology Letters 243 (1), 107–115.

Duchêne, E., Legras, J., Karst, F., Merdinoglu, D., Claudel, P., Jaegli, N., Pelsy, F., 2009. Variation of linalool and geraniol content within two pairs of aromatic and non-aromatic grapevine clones. Australian Journal of Grape and Wine Research 15 (2), 120–130.

Fernández-González, M., Di Stefano, R., Briones, A., 2003. Hydrolysis and transformation of terpene glycosides from muscat must by different yeast species. Food Microbiology 20 (1), 35–41.

Ferreira, V., Pena, C., Escudero, A., Cacho, J., 1996. Losses of volatile compounds during fermentation. Zeitschrift für Lebensmittel-Untersuchung und-Forschung 202, 318–323.

Fia, G., Giovani, G., Rosi, I., 2005. Study of beta-glucosidase production by wine-related yeasts during alcoholic fermentation. A new rapid fluorimetric method to determine enzymatic activity. Journal of Applied Microbiology 99 (3), 509–517.

Fitzpatrick, A.H., Bhandari, J., Crowell, D.N., 2011. Farnesol kinase is involved in farnesol metabolism, ABA signaling and flower development in Arabidopsis. The Plant Journal for Cell and Molecular Biology 66 (6), 1078–1088.

Francis, I.L., Newton, J.L., 2005. Determining wine aroma from compositional data. Australian Journal of Grape and Wine Research 11, 114–126.

Gamero, A., Manzanares, P., Querol, A., Belloch, C., 2011. Monoterpene alcohols release and bioconversion by Saccharomyces species and hybrids. International Journal of Food Microbiology 145 (1), 92–97.

Gramatica, P., Manitto, P., Ranzi, B.M., Delbianco, A., Francavilla, M., 1982. Stereospecific reduction of geraniol to (R)-(+)-citronellol by *Saccharomyces cerevisiae*. Experientia 38 (7), 775–776.

Guth, H., 1997. Quantitation and sensory studies of character impact odorants of different white wine varieties. Journal of Agricultural and Food Chemistry 45 (8), 3027–3032.

HSDB, Hazardous Substances data Base. http://toxnet.nlm.nih.gov.

King, A., Dickinson, J.R., 2000. Biotransformation of monoterpene alcohols by *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* and *Kluyveromyces lactis*. Yeast 16 (6), 499–506.

King, A., Dickinson, J.R., 2003. Biotransformation of hop aroma terpenoids by ale and lager yeasts. FEMS Yeast Research 3, 53–62.

Luan, F., Wüst, M., 2002. Differential incorporation of 1-deoxy-D-xylulose into (3S)-linalool and geraniol in grape berry exocarp and mesocarp. Phytochemistry 60 (5), 451–459.

Mason, A.B., Dufour, J.P., 2000. Alcohol acetyltransferases and the significance of ester synthesis in yeast. Yeast 16 (14), 1287–1298.

Morakul, S., Mouret, J.R., Nicolle, P., Aguerra, E., Sablayrolles, J.M., Athès, V., 2012. A dynamic analysis of higher alcohols and ester release during winemaking fermentations. Food Bioprocess Technology. http://dx.doi.org/10.1007/s11947-012-0827-4.

Novo, M., Bigey, F., Beyne, E., Galeote, V., Gavory, F., Mallet, S., Cambon, B., et al., 2009. Eukaryote-to-eukaryote gene transfer events revealed by the genome sequence of the wine yeast *Saccharomyces cerevisiae* EC1118. Proceedings of the National Academy of Sciences of the United States of America 106 (38), 16333–16338.

Odat, O., Matta, S., Khalil, H., Kampranis, S.C., Pfau, R., Tsichlis, P.N., Makris, A.M., 2007. Old yellow enzymes, highly homologous FMN oxidoreductases with modulating roles in oxidative stress and programmed cell death in yeast. The Journal of Biological Chemistry 282 (49), 36010–36023.

Oswald, M., Fischer, M., Dirninger, N., Karst, F., 2007. Monoterpenoid biosynthesis in *Saccharomyces cerevisiae*. FEMS Yeast Research 7 (3), 413–421.

Ribereau-Gayon, P., Boidron, J.N., Terrier, A., 1975. Aroma of Muscat grape varieties. Journal of Agricultural and Food Chemistry 23 (6), 1042–1047.

Schreier, P., Jennings, W.G., 1979. Flavor composition of wines: a review. Critical Reviews in Food Science & Nutrition 12 (1), 59–111.

Simpson, R., Miller, G., 1983. Aroma composition of aged Riesling wine. American Journal of Enology and Viticulture 22, 51–63.

Stucky, G.J., McDaniel, M.R., 1997. Raw hop aroma qualities by trained panel free-choice profiling. Journal of the American Society of Brewing Chemists 55 (2), 65–72.

Takoi, K., Itoga, Y., Koie, K., Kosugi, T., 2010. The contribution of geraniol metabolism to the citrus flavour of beer: synergy of geraniol and β-citronellol under coexistence with excess linalool. Journal of the Institute of Brewing 116 (3), 251–260.

Trotter, E.W., Collinson, E.J., Dawes, I.W., Grant, C.M., 2006. Old yellow enzymes protect against acrolein toxicity in the yeast *Saccharomyces cerevisiae*. Applied and Environmental Microbiology 72 (7), 4885–4892.

Ugliano, M., Bartowsky, E.J., McCarthy, J., Moio, L., Henschke, P.A., 2006. Hydrolysis and transformation of grape glycosidically bound volatile compounds during fermentation with three Saccharomyces yeast strains. Journal of Agricultural and Food Chemistry 54 (17), 6322–6331.

van der Merwe, C.A., van Wyk, C.J., 1981. The contribution of some fermentation products to the odor of dry white wines. American Journal of Enology and Viticulture 32 (1), 41–46.
Vaudano, E., Moruno, E.G., Di Stefano, R., 2004. Modulation of geraniol metabolism during alcohol fermentation. Journal of the Institute of Brewing 110 (3), 213–219.
Vaz, A.D.N., Chakraborty, S., Massey, V., 1995. Old yellow enzyme: aromatization of cyclic enones and the mechanism of a novel dismutation reaction. Biochemistry 34 (13), 4246–4256.
Verstrepen, K.J., Van Laere, S.D.M., Vanderhaegen, B.M.P., Derdelinckx, G., Dufour, J.P., Pretorius, I.S., Winderickx, J, 2003. Expression levels of the yeast alcohol acetyltransferase genes ATF1, Lg-ATF1, and ATF2 control the formation of a broad range of volatile esters. Applied and Environmental Microbiology 69, 5228–5237.
Warburg, O., Christian, W., 1933. Über das gelbe ferment und seine wirkungen. Biochemische Zeitschrift 266, 377–411.
Yamamoto, T., Shimada, A., Ohmoto, T., Matsuda, H., Ogura, M., Kanisawa, T., 2004. Olfactory study on optically active citronellyl derivatives. Flavour and Fragrance Journal 19, 121–133.
Yuan, T., Chen, Q., Zhao, P., Zeng, Y., Liu, X., Lu, S., 2011. Identification of enzymes responsible for the reduction of geraniol to citronellol. Natural Products and Bioprocessing 1, 108–111.
Zoecklein, B., Marcy, J., Williams, J., Jasinski, Y., 1997. Effect of native yeasts and selected strains of *Saccharomyces cerevisiae* on glycosyl glucose, potential volatile terpenes, and selected aglycones of White Riesling (*Vitis vinifer*a L.) wines. Journal of Food Composition and Analysis 10 (1), 55–65.