De novo synthesis of monoterpenes by \textit{Saccharomyces cerevisiae} wine yeasts

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Abstract

This paper reports the production of monoterpenes, which elicit a floral aroma in wine, by strains of the yeast \textit{Saccharomyces cerevisiae}. Terpenes, which are typical components of the essential oils of flowers and fruits, are also present as free and glycosylated conjugates amongst the secondary metabolites of certain wine grape varieties of \textit{Vitis vinifera}. Hence, when these compounds are present in wine they are considered to originate from grape and not fermentation. However, the biosynthesis of monoterpenes by \textit{S. cerevisiae} in the absence of grape derived precursors is shown here to be of de novo origin in wine yeast strains. Higher concentration of assimilable nitrogen increased accumulation of linalool and citronellol. Microaerobic compared with anaerobic conditions favored terpene accumulation in the ferment. The amount of linalool produced by some strains of \textit{S. cerevisiae} could be of sensory importance in wine production. These unexpected results are discussed in relation to the known sterol biosynthetic pathway and to an alternative pathway for terpene biosynthesis not previously described in yeast.

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1. Introduction

The significance of volatile monoterpenes to the flavor and varietal character of some cultivars of \textit{Vitis vinifera} grapes is well reviewed [1–3]. Apart from the hitherto known compounds (terpene ethers, monoterpene alcohols) numerous monoterpene compounds, in particular monoterpene diols, have been identified in grape must and wine. The dominating monoterpenes alcohol found in several Muscat-type varieties and others like: Gewürztraminer, Riesling or Chardonnay, are linalool, geraniol, nerol, citronellol, and α-terpineol [3].

The formation of terpenes by yeasts is limited to trace concentrations by a small number of non-\textit{Saccharomyces} species: \textit{Kluveromyces lactis} [4], \textit{Torulaspora delbrueckii} [5], \textit{Kloeckera apiculata}, \textit{Metschnikowia pulcherrima} and \textit{Candida stellata} [6], and \textit{Ambrosiozyma monospora} [7]. Hock et al. [6] concluded that \textit{Saccharomyces cerevisiae}, only produced approximately 2 μg l\textsuperscript{-1}
of farnesol, a hydrolysis product of the ergosterol precursor farnesyl pyrophosphate. The formation of terpenes by *S. cerevisiae* in grape musts, was proposed [8], but in this report the amount of glycosylated precursor compounds present in the juice and companion wine was not measured. The reported increase in the amount of free geraniol and linalool after fermentation, is a well known phenomenon during winemaking that could be due to the β-glucosidase activity of yeasts and/or chemical hydrolysis of the bound forms [9–12].

Lynen proposed in 1964 [13] that terpene and sterol biosynthesis are related. Anaerobic conditions were suggested to inhibit several essential steps in ergosterol biosynthesis, including squalene epoxidation and the oxidative demethylation/dehydrogenation of lanosterol, essential steps for the formation of ergosterol. Some researchers suggested that such inhibition of sterol biosynthesis could stimulate terpene formation by fungi due to the accumulation of sterol precursor compounds [14]. Alternatively, interrupting the sterol pathway by mutation can alter terpene content. *S. cerevisiae* farnesyl-diphosphate synthase (ERG20) mutants accumulate high levels of geraniol [15]. However, the de novo formation of monoterpenes by natural strains of *S. cerevisiae* has not been reported. The demonstration that exogenous monoterpenes can undergo biotransformation provides further evidence for the presence of a terpene metabolic pathway in yeast. *S. cerevisiae* can biotransform geraniol to citronellol in winemaking conditions [16]. Biotransformation of the monoterpenes linalool, α-terpineol, nerol and geraniol by *S. cerevisiae* was recently shown in model fermentations [17]. Total terpene content did not increase in these biotransformations, the mechanisms of which are currently ill-defined. Furthermore, only one cyclase gene related to sterol metabolism in yeast, lanosterol synthase, has been reported [18]. Isoprenoids play many roles in human health, and plant and animal physiology [19–21]. Therefore, a better understanding of isoprenoid biosynthetic pathways in the model unicellular eukaryote, *S. cerevisiae*, could provide invaluable clues for understanding systems that are more complex. However, as fungi do not typically produce monoterpenes, research on the synthesis pathways has been focused on polyterpenoids such as triterpenoids, carotenoids and ubiquinone [22]. There are no studies in yeasts specifically concerning monoterpenes. The biosynthesis of sterols and some diterpenes and triterpenes in fungi and yeasts have been described to proceed by the mevalonic acid (MVA) pathway, through isopentenyl pyrophosphate (IPP) and dimethyl allyl pyrophosphate (DMAPP) as intermediates (building blocks) [14,22–26]. However, recently it has been shown in plants that terpene biosynthesis follows two pathways in different cellular compartments [27]. The MVA pathway, that is carried out in the cytoplasm, as in fungi, and a new terpene biosynthetic pathway, the MEP pathway (2-C-methyl-d-erythritol 4-phosphate pathway), that was discovered in bacteria in 1993 [28], has been shown to occur in the plastid organelles of higher plants [27,29]. Interestingly, this pathway has been proposed to be used exclusively in plants for the formation of monoterprenes [30]. However, no close homologues were found with the MEP pathway key enzyme, 1-deoxyxylulose-5-phosphate reductoisomerase (DXR) of plants in fungi [31].

A third pathway interconnecting leucine catabolism and isoprenoid metabolism has been studied in fungi [24,25]. Although the precise enzymatic basis for the link between sterol biosynthesis and leucine catabolism is still lacking, there is strong genetic evidence recently reported for the fungus *Aspergillus nidulans* [32], that supports the existence of such a metabolic link. The pivotal reaction required for both leucine catabolism and isoprenoid metabolism is catalyzed by the enzyme 3-methylcrotonyl-CoA carboxylase (MCCase; EC 6.4.1.4) that has been described in some bacterial species and is present in the mitochondria of mammals and plants [33,34]. There are no studies specifically concerning the existence of MCCase in yeast.

During fermentation of an aromatic grape juice (*Vitis vinifera* cv. Moscato Giallo) with mixed cultures of non-*Saccharomyces* and *S. cerevisiae* strains, we detected approximately 30% increase in terpene concentration based on the total amount of free and conjugated terpenes analyzed before and after fermentation [35]. The aim of the present study, was to conclusively test for de novo production of terpenes. Several strains of *S. cerevisiae* and one *Hanseniaspora uvarum* were tested in parallel model fermentations in a simple chemically defined medium, lacking of grape juice, terpenes or their glycoconjugates. The widely studied wine yeast strain, *S. cerevisiae* M522 (University of California, Davis) was used to evaluate the factors affecting terpene production. The de novo biosynthesis of monoterpenes by *S. cerevisiae* is discussed in relation to the known isoprenoid pathways in yeast and other organisms.

2. Material and methods

2.1. Yeast strains

*Saccharomyces cerevisiae* strains utilized were: Montrachet 522 (University of California, Davis), CIVC 8130 (France), and three Uruguayan native isolates from our collection, 881, 882 and KU1 [36], all of which are used in the commercial production of wine. A Uruguayan native strain of *Hanseniaspora uvarum*, isolated from grape juice [35], was utilized as a non-*Saccharomyces* control. Inocula were prepared in the same synthetic medium by incubation for 12 h in a rotary shaker at 150 rpm and 25 °C. Inoculum size was 5 × 10^5 cells ml⁻¹ of
medium for all the strains. The wine yeast strain, *S. cerevisiae* M522 [37] was used to evaluate the factors affecting terpene production.

2.2. Fermentation conditions

Chemically defined fermentation medium (resembling grape juice composition) was prepared as previously described but lacking grape derived secondary metabolites [38], and modified [35] as follows: nitrogen content was adjusted for a yeast assimilable nitrogen (YAN) level (sum of amino acids and ammonium without proline in the same proportions indicated previously [38]) of 50 mgN l\(^{-1}\). Media with YAN levels of 180 and 400 mgN \(l^{-1}\) were made by supplementation with diammonium phosphate. The latter two YAN levels are not limiting for a normal complete fermentation by the yeast strains utilized and are common levels in grape juice. The final pH of each medium was adjusted to 3.5 with acid or alkaline. Equimolar concentration of glucose/fructose were adjusted to 120 g \(l^{-1}\), although this is a smaller concentration than in grape juice, it was shown suitable for both *Saccharomyces* and non-*Saccharomyces* strains preventing alcohol inhibition. It was not found necessary for complete fermentation and it has a negative impact on the sensory characteristics of the Resultant wines.

Fermentations were carried out in 125 ml of medium contained in 250 ml Erlenmeyer flasks, closed with either cotton wool plugs to achieve Microaerobic conditions (Microaer) or Muller valves, filled with pure sulfuric acid, to achieve anaerobic conditions (Anaer). YAN and redox potential were chosen as variables for this investigation since it is known that these factors significantly affect other fermentation aroma compounds formed by yeast in the same experimental conditions [35]. Static batch fermentations were conducted at 20 °C in duplicate, simulating wine making conditions.

2.3. GC–MS analysis

Extraction of aroma compounds (50 ml samples) was performed by adsorption and separate elution from an Isolute (IST Ltd., Mid Glamorgan, UK) ENV+ cartridge packed with 1 g of highly cross-linked styrene-divinyl benzene (SDVB) polymer. The terpenes were eluted with 30 ml of dichloromethane; the solution was dried with Na\(_2\)SO\(_4\) and concentrated to 1.5 ml on a Vigreux column, stored at –10 °C, and, immediately prior to HRGC-MS analysis, further concentrated to 100 \(\mu\)l under a gentle nitrogen stream. Terpene analysis were performed in an HRGC-MS Turbomass Gold spectrometer Perkin–Elmer. The experimental conditions were as follow: capillary column, DBWax (60 m × 0.32 mm i.d., 0.5 \(\mu\)m film thickness); carrier gas, He (4 \(ml\) min\(^{-1}\)); oven temperature program, 50 °C at 2 °C min\(^{-1}\) to 220 °C, then 220 °C for 15 min. Injector temperature, 250 °C; injection mode, splitless; volume injected 1.0 \(\mu\)l. Helium was used as a carrier (4 \(ml\) min\(^{-1}\)); interface temperature, 250 °C; TIC acquisition mass range, TIC \(m/z\) 30–300; scan time, 0.2 s; inter-scan delay, 0.01 s. Compounds were evaluated as TIC peaks area ratio with respect to 1-heptanol with RF = 1; terpenes were evaluated using the following fragments from contemporary SIR and SCAN processing, and putting them in relation to the sum of all fragments from a reference spectrum obtained eluting standards: oxide A (*trans*-linalool oxide, furanoid) (\(ml/z\) 59, 94, 111) as 28.7% TIC; oxide B (*cis*-linalool oxide, furanoid), (\(ml/z\) 59, 94, 111) as 27.8% TIC; linalool (\(ml/z\) 71, 93, 121) as 25.7% TIC; geranyl (3,7-dimethyl-1,5,7-octatrien-3-ol) (\(ml/z\) 71, 81) as 26.8% TIC, α-terpineol (\(ml/z\) 59, 93, 121, 136) as 39.7% TIC, oxide C (*trans*-linalool oxide, pyranoid) (\(ml/z\) 59, 68, 94) as 38.7% TIC; oxide D (*cis*-linalool oxide, pyranoid) (\(ml/z\) 59, 68, 94) as 38.3% TIC; citronellol (\(ml/z\) 81, 95, 109, 123) as 16.3% TIC, nerol (\(ml/z\) 69, 93, 111, 121) as 28.7% TIC; geraniol (\(ml/z\) 93, 121) as 4.2% TIC.

2.4. Blast searches for putative protein homologues related with monoterpenes

Only protein sequences corresponding to enzymes with established biochemical function were used for similarity searches of *DXR*, *MCC* and *Geranyl diphosphate* synthase (GPP). Protein sequences were extracted from websites (www.ncbi.nlm.nih.gov; www.arabidopsis.org) and used to perform tblastn and blastp similarity searches against the complete DNA sequence of *S. cerevisiae* and the set of verified ORFs, respectively, using the blast tools provided on the *Saccharomyces* Genome Database website (www.yeastgenome.org). Multiple protein alignments were performed on the website http://www.ebi.ac.uk/clustalw/ changing the matrix parameter to blosum30. Conserved domains were analyzed at NCBI and ProDom (http://prodes.toulouse.inra.fr/prodom/current/html/home.php) websites.

2.5. Statistical analysis

ANOVA for ammonium, redox condition, duplicates and for each strain was made to determined the significant differences of the behaviour of monoterpenes formation. Concentration differences of free volatile compounds were evaluated, mean rating and least significant differences for each term were calculated from an analysis of variance. Statistica 5.1. software was utilized for the analysis and graphs construction.
3. Results and discussion

3.1. Biosynthesis of monoterpenes by *Saccharomyces cerevisiae*

The intraspecific variability of *Saccharomyces cerevisiae* strains to accumulate terpenes in a chemically artificial grape medium was determined. The results presented in Fig. 1, clearly show that linalool and α-terpineol are the terpenes produced in greatest abundance by all the *S. cerevisiae* wine yeasts tested. Whereas *H. uvarum* also produced similar concentrations of linalool and α-terpineol, little citronellol was formed despite a relatively high amount of geraniol being present. This occurrence could be explained by the inability of this strain to reduce geraniol to citronellol, as has been described for *Torulaspora delbrueckii* species when compared to *Saccharomyces* strains during biotransformation experiments [17]. The amount of linalool produced by strain KU1 could be of sensory importance in wine production, with concentrations approaching the threshold value for this compound (5 μg l⁻¹). Given that this strain is considered one of the best low nitrogen demanding yeast in relation to fermentation aroma compounds produced [35], it was considered of interest to obtain a better understanding of terpene biosynthesis by industrial yeasts in relation to nitrogen metabolism. Other terpenes present in trace amounts and detected in concentrations lower than 0.5 μg l⁻¹ for all the strains, were the following: nerol, trans (Oxide A) and cis (Oxide B) furan linalool oxides, trans (Oxide C) and cis (Oxide D) pyran linalool oxides and 3,7-dimethyl-1,5,7-octatrien-3-ol (Ho-trienol).

3.2. Effect of nitrogen and redox conditions

The wine yeast strain, *S. cerevisiae* M522 was used to evaluate the factors affecting terpene production. The effect of nitrogen and redox conditions in the chemically defined medium was studied at two levels of assimilable nitrogen (180 and 400 mgN l⁻¹) and under two redox conditions: microaerobic and anaerobic; since it is known that these factors significantly affect other fermentation aroma compounds formed by yeast in the same experimental conditions [35]. The highest concentration of terpenes was obtained under conditions that stimulated glycolytic flux. Microaerobic and high assimilable nitrogen in the synthetic media favored the highest terpene accumulation of approximately 16 μg l⁻¹, composed mainly of linalool, α-terpineol, citronellol and geraniol (Fig. 2).

A significant positive effect of the ammonium level, on the synthesis of linalool under the two redox conditions analyzed (p < 0.001), with a trend favored by microaerobic conditions, is shown in Fig. 2(a). It could be seen that linalool synthesis was more clearly promoted by microaerobic conditions (p < 0.01). This condition increased fermentation activity, but did not significantly affect total cell population [35]. These results suggest that the sterol biosynthetic pathway does not exclusively lead to the formation of terpenes, and that glycolysis may contribute.

In the case of citronellol, Fig. 2(b) shows that its accumulation in the medium was only influenced by assimilable nitrogen supplementation under microaerobic conditions. A similar result has been observed with *Kluyveromyces lactis* supplemented with asparagine, as the nitrogen source, under aerobic conditions [4]. On the other hand, α-terpineol, which was produced in highest concentration under these conditions (up to 11 μg l⁻¹), is considered to be one of the main products of chemical [39] and/or biological transformation of linalool by *Saccharomyces* [17]. In the present study, ammonium and redox conditions did not affect α-terpineol formation (data not shown), probably suggesting that the low pH of the medium led to its formation by

![Fig. 1. Biosynthesis of monoterpenes by strains of *Saccharomyces cerevisiae* (Sc) and one strain of *Hanseniaspora uvarum* during fermentation of the chemically defined medium containing a yeast assimilable nitrogen (YAN) of 400 mgN l⁻¹. Standard deviation of the mean is indicated with error bars.](image-url)
the chemical transformation of linalool, rather than the involvement of a cyclase enzyme. In the case of geraniol, no significant effects were found either (data not shown). This compound is the likely precursor for the formation of citronellol, linalool and nerol (Fig. 4), and this could explain the observed behavior.

Nerolidol, is a sesquiterpene alcohol derived from farnesyl pyrophosphate (Fig. 3(a)), and its production was clearly influenced by redox situation at the lower level of nitrogen, which was positively affected by anaerobic conditions. As expected from studies on the biosynthesis of sterols by yeast [14], nerolidol accumulation could be stimulated by the inhibition of sterol biosynthesis induced by low nitrogen combined with anaerobic conditions. In relation to farnesol, Fig. 3(b), shows that there was a significant effect with nitrogen level ($p < 0.01$), but not with the redox condition. Because farnesol-PP is an intermediary of sterol biosynthesis and it is a precursor of nerolidol formation, the lowered concentration of farnesol could be explained by the presence of nitrogen which stimulates its metabolism by one of the two mechanisms depending on the redox condition of the medium (see the scheme of Fig. 4). Under anaerobic conditions, nerolidol formation would be stimulated, whereas under microaerobic conditions the sterol biosynthesis pathway would be stimulated.
Interestingly, very few studies are found in the literature regarding the regulation of isoprenoids by nitrogen. Recently, the biosynthesis of the diterpene gibberellin by the fungus *Gibberella fujikuroi* \[40\] was shown to be decreased by ammonium. The behavior of sesquiterpene alcohols in our study agrees with this report, but the monoterpenes showed the opposite behavior.

In summary, the behavior found in this work for the formation of monoterpenes during fermentation is not consistent with the sterol biosynthetic pathway described in yeast. These results suggest the existence of an independent pathway for their formation, possibly located in a different cell compartment. On the other hand, the behavior found with the sesquiterpene alcohols, nerolidol and farnesol in these experiments is in agreement with the cytosolic sterol metabolic pathway already described in yeast. The different behaviour of mono- and sesquiterpenes in our study taken together with the fact that high nitrogen and microaerobic conditions increased fermentation activity but not total cell population, led us to explore the possibility of alternative pathways that could account for the behaviour of monoterpenes in *Saccharomyces cerevisiae*, as has been demonstrated in plant cells \[27,29\]. Although a compartmentalized sterol biosynthetic pathway was proposed in *S. cerevisiae* in relation to the isozymes 3-methylglutaryl CoA reductases, HMGR1 and HMGR2 \[41\] (see Fig. 4), it is not clear how a monoterpenes pathway could share one of these compartments.

3.3. Origin of monoterpenes in yeast and blast searches

To confirm whether the MEP pathway exist in *S. cerevisiae*, we searched for protein sequences showing homology to the key enzyme of the MEP pathway, DXR, recently identified in plant and eubacteria. However, no close homologues were found, confirming the previous report \[31\], and supporting the notion that this pathway would not be functional in yeast \[22\].

However, although experimental demonstration for the existence of the leucine catabolic pathway in
S. cerevisiae is needed, the results of blast searches showed significant homology of well studied MCCases with several proteins in S. cerevisiae. Both tblastn and blastp searches of the S. cerevisiae genome with either a consensus protein sequence of MCCα (from human and Arabidopsis origin) or the MCCα protein sequence from Aspergillus nidulans revealed the same high-scoring (e-values of <10<sup>-37</sup>) sequences corresponding to DUR1,2; PYC2; PYC1; HFA1 and ACC1. From this group of proteins only HFA1 is localized in the mitochondrion in eukaryotic cells.
mitochondria. YMR207C/HFA1 codes for a protein of unknown function; a deletion mutant in this gene is viable but shows respiratory defects [42]. On the other hand, the geranyl diphosphate synthase (GPPase) is another key enzyme for monoterpene synthesis, that produces geranyl diphosphate from the IPP pool (see Fig. 4), known to localize in plant plastids. Although not previously described in yeast, our search also gave significant homology of Arabidopsis GPP synthase with another yeast mitochondrial precursor, the hexaprenyl pyrophosphate synthetase (HPS, COQ 1). GPPase from Arabidopsis thaliana showed 36% identical aminoacids and 59% conservative substitutions with the hexaprenyl pyrophosphate synthetase (HPS, COQ 1) of Saccharomyces cerevisiae. The known function of this enzyme catalyzes the first step in ubiquinone (coenzyme Q) biosynthesis in yeast.

3.4. “MCC pathway”: a model for the formation of monoterpenes in S. cerevisiae

In Fig. 4, we propose a hypothetical model for the formation of monoterpenes in S. cerevisiae, probably occurring in the mitochondrion and related to leucine metabolism that could account for the stimulation of their formation by microaerobic conditions and high assimilable nitrogen in a chemically defined medium. This scheme depicts possible mechanisms for the formation of the monoterpenes detected in this work. The putative GPP synthase of mitochondrial localization identified in this work in the Saccharomyces cerevisiae genome, argues in favor of this hypothesis. These separate pathways (one in mitochondria and the other in cytosol) would explain the differences found in the present work between monoterpenes formation and sesquiterpene metabolism. Furthermore, this model could also explain the report of Casey et al. [41], about HMGR1 and HMGR2 isoymes in S. cerevisiae, the formation of geraniol by erg 20 mutants [15] and the proposed compartmentation of the MVA pathway for the production of carotenoids and sterols in fungal cells [22,43,44].

Preliminary studies with a wild laboratory strain (S. cerevisiae AB 972) gave only traces of monoterpenes formation in the experimental conditions of this work (data not shown), making still difficult to conclusively test this pathway with available mutants. Interestingly, Industrial wine yeast strains have a much more complex genome (polyploid or aneuploid) in comparison with the completely sequenced haploid laboratory strain and therefore, we cannot rule out the existence of proteins adapted for monoterpenes metabolism as there are for industrial wine fermentation. It is difficult to delete all copies of these genes in aneuploid industrial strains that are genetically not well characterized. Future research must be focus in the exploration of other haploid S. cerevisiae strains, to demonstrate this hypothesis with mutants.

Finally, this report also shows a new concept in relation to wine aroma, questioning the origin of grape Vitis vinifera aroma compounds by the demonstration that yeast may indeed contribute to the monoterpene composition of a wine.

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