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Short communication

Overproduction of isoprenoids by *Saccharomyces cerevisiae* in a synthetic grape juice medium in the absence of plant genes

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ABSTRACT

The objective of this work is to demonstrate if the hexaprenyl pyrophosphate synthetase Coq1p might be involved in monoterpenes synthesis in *Saccharomyces cerevisiae*, although its currently known function in yeast is to catalyze the first step in ubiquinone biosynthesis. However, in a BY4743 laboratory strain, the presence of an empty plasmid in a chemically defined grape juice medium results in a statistically significant increase of linalool, (*E*)-nerolidol and (*E,E*)-farnesol. When *COQ1* is overexpressed from a plasmid, the levels of the volatile isoprenoids are further increased. Furthermore, overexpression of *COQ1* in the same genetic context but with a mutated farnesyl pyrophosphate synthetase (*erg20* mutation K197E), results in statistically significant higher levels of linalool (above 750 μg/L), geraniol, α-terpineol, and the sesquiterpenes, farnesol and nerolidol (total concentration of volatile isoprenoids surpasses 1300 μg/L). We show that the levels of monoterpenes and sesquiterpenes that *S. cerevisiae* can produce, in the absence of plant genes, depend on the composition of the medium and the genetic context. To the best of our knowledge, this is the highest level of linalool produced by *S. cerevisiae* up to now. Further research will be needed for understanding how *COQ1* and the medium composition might interact to increase flavor complexity of fermented beverages.

1. Introduction

Volatile isoprenoids are extensively used in the production of food additives and preservatives. Consequently, much work has focused on the heterologous expression of plant terpene synthases in *Saccharomyces cerevisiae*, to improve the production yield and efficiency. In many instances, the heterologous expression of plant terpene synthases yields low levels of isoprenoids in yeast, particularly monoterpenes (Vickers et al., 2014). However, more recently, the successful expression of a plant geranyl synthase resulted in high concentrations of geraniol in a wine yeast (Pardo et al., 2015).

Terpenoids or isoprenoids are naturally occurring compounds synthesized from five-carbon isoprene units [isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP)], assembled and modified in thousands of ways. There are over 40,000 different isoprenoids with markedly diverse functions that are classified according to the number of carbon atoms of the molecule. Examples include monoterpenes (C10) and sesquiterpenes (C15) (Bohlmann and Keeling, 2008), compounds with impact on the fruity and flowery flavor profiles of plants. There are present in diverse human-consumed food products such as fruits, juice, wine, mushrooms, spices, beer, tea and coffee, among others (Maarse, 1991). The aroma threshold of these compounds, such as linalool (10 μg/L), geraniol (40 μg/L), nerolidol (100 μg/L), and farnesol (100 μg/L) are moderately low in wine (Guth, 1997; Li et al., 2008). Nonetheless, they contribute significantly to the sensory perception, mainly in aromatic grape (*Vitis vinifera*) varieties, when they are in the free form, not bound to sugars (Guth, 1997; Li et al., 2008). In nature, some volatile isoprenoids function as insect attractants and repellents, are synthesized as abiotic stress protectants, and also serve as antimicrobials in pathogen resistance. The production of these volatile compounds is inducible and regulated at appropriate levels and timing, as well as compartmentalized within the cell, to avoid interference with normal metabolism (Loreto et al., 2014; Vickers et al., 2014).

Plants produce IPP and DMAPP using two independent pathways: the primarily cytosolic mevalonic acid pathway and the plastidial

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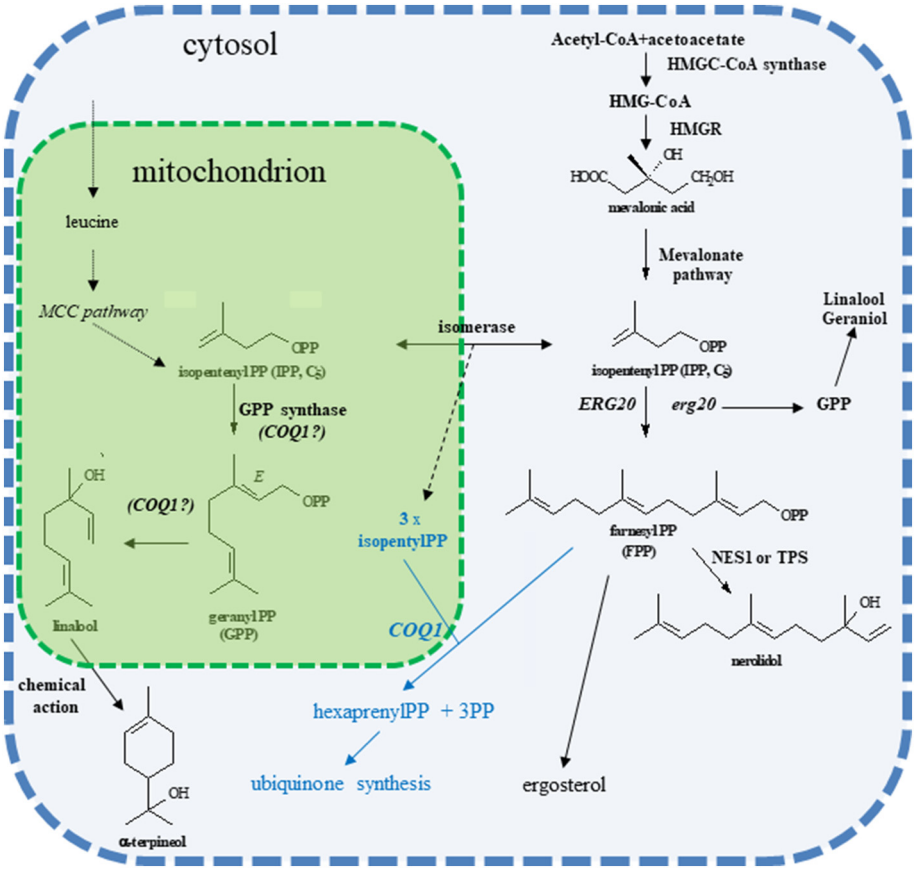


Fig. 1. Metabolic pathways of monoterpenes and sesquiterpenes synthesis in yeast. The compounds that are analyzed in this work are shown here: linalool, geraniol, α-terpineol, nerolidol, and farnesol. Genes are indicated for each pathway. The *erg20* mutant effects and the *COQ1* known function (in blue) for ubiquinone synthesis are shown. It is known that low pH medium, such as the chemically defined grape juice medium (CDGJM) utilized here (pH 3.5), increases chemical conversion of geraniol to linalool (Fischer et al., 2011) and linalool to α-terpineol (Williams et al., 1982). *COQ1*, with question marks, is the hypothetical function proposed by Carrau et al. (2005). This scheme also depicts a potential effect of phosphorus demand or supply, and the leucine 3-methylcrotonyl-coenzyme A carboxylase (MCC) pathway that might be affecting the different pathways related to monoterpenes and sesquiterpenes formation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

methylerythritol phosphate pathway (Tholl, 2015). In fungi and animals, only the mevalonic acid pathway is present and, except in some insects (Gilg et al., 2005), no monoterpene synthases have been reported. Monoterpenes have only rarely been reported from fungi (Huang et al., 2006; Lanza and Palmer, 1977; Lin et al., 2013) and some yeasts (Carrau et al., 2005). Volatile sesquiterpenes are widely distributed in many plants essential oils, in animals, and microbes. In yeast, such as *Candida* species, they have been reported as cell-cell signaling molecules: (*E,E*)-farnesol for *Candida albicans* and (*E*)-nerolidol for *Candida parapsilosis* (Hornby et al., 2001; Martins et al., 2010). Farnesol is produced by dephosphorylation of farnesyl diphosphate (FPP) by phosphatases, pyrophosphatases or specific sesquiterpene synthases (Wang et al., 2011). A yeast strain of *Rhodotorula glutinis* has been shown to produce nerolidol (Nishino et al., 1982).

In yeast, there is a unique enzyme Erg20p that synthesizes FPP from isoprene precursors. Deletion of *ERG20* is lethal because mutants are unable to synthesize ergosterol (Fig. 1), an essential component of cell membranes required to maintain membrane permeability and fluidity (Anderson et al., 1989). In an *erg20* point mutant context (K197E), the tight binding of geranyl diphosphate (GPP) to the farnesyl diphosphate synthase catalytic site is relaxed, and GPP is freely available as a precursor for the synthesis of the monoterpenes, geraniol, and linalool (Fischer et al., 2011). Originally, it was thought that there is no free GPP in yeast. However, we have demonstrated that native wine strains can synthesize monoterpenes *de novo* in a chemically defined grape juice medium (CDGJM) that does not contain any plant precursor, such as glycosylated monoterpenes or higher alcohols (Carrau et al., 2005). Furthermore, it was reported that there is naturally a small pool of free intracellular GPP in yeast (Oswald et al., 2007).

Mutations in the FPP synthase gene (*ERG20*) increase accumulation of geraniol in yeasts (Chambon et al., 1990; Blanchard and Karst, 1993), and formation is further increased in a medium with yeast assimilable nitrogen (YAN) and oxygen availability during fermentation (Carrau et al., 2005). It is currently thought that GPP is dephosphorylated by non-specific phosphatases/pyrophosphatases to make monoterpene alcohols (Oswald et al., 2007). However, it has also been shown that FPP synthase has pyrophosphatase activity (Fischer et al., 2011).

We proposed that the mitochondrial hexaprenyl pyrophosphate synthetase Coq1p (Fig. 1), which showed 36% identical amino acids and 59% conservative substitutions with the plant geranyl diphosphate synthase of *Arabidopsis thaliana*, might be involved in isoprenoid biosynthesis. Its currently known function in yeast is to catalyze the first step in coenzyme Q biosynthesis (Gin and Clarke, 2005). More recently, however, results presented by Huang et al. (2013) showed that *COQ1* disruption could be associated with isoprenoid biosynthesis and mitochondrial functions. Furthermore, a very recent functional divergence analysis suggested that different types of sub-functionalization in the Coq1 gene family have occurred in animals and fungi compared to plants (Zhao et al., 2017).

In this work, we show that depending on the composition of the medium and the genetic context, *S. cerevisiae* can produce significant increased levels of the monoterpene linalool (sensory described as floral, coriander, bergamot, lavender) and the sesquiterpenes, *E*-nerolidol (floral, fruity, orange) and *E,E*-farnesol (lemon, anise, peach, raspberry), when *COQ1* is overexpressed from a plasmid. The impact on flavor diversity in fermented beverages should be significant from the sensory perspective, according to the concentrations obtained, which are above the threshold values.

2. Materials and methods

2.1. Yeast strains and plasmids

Saccharomyces cerevisiae BY4743 (*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0*) and K197E

(*erg20* mutant constructed and described by Fischer et al., 2011, derived from BY4743) were used in this study.

Plasmids pADCL, pHA1-1, pRS316, and pRSQ1-1 are described in Gin and Clarke (2005). *S. cerevisiae COQ1* was cloned in pRS316 under the control of the native *COQ1* promoter, and the resulting plasmid was named pRSQ1-1. *S. cerevisiae COQ1* was cloned in pADCL under the control of the *ADH1* constitutive promoter and named pHA1-1.

2.2. Culture media and growth conditions

Chemically defined grape juice medium (CDGJM) was prepared with glucose (60 g/L) and fructose (60 g/L), a lower level than in grape juices to minimize potential interference that might be caused by ethanol and CO_2, as it was previously observed by Carrau et al. (2005) for some yeast strain screenings. Isoprenoid production in this model medium at both sugar levels (120 and 200 g/L) was confirmed peaking at 72 h of culture and then declining. Medium was completed with K_2HPO_4 (1.14 g/L), $MgSO_4.7H_2O$ (1.23 g/L), $CaCl_2.2H_2O$ (0.44 g/L), potassium hydrogen tartrate (2.5 g/L), malic acid (3 g/L), citric acid (0.2 g/L), $(NH_4)_2HPO_4$ (0.4 g/L), vitamin solution (10 mL/L of a 100× stock solution), amino acids solution (10 mL/L of a 100× stock solution), microelements solution (1 mL/L of a 1000× stock solution), and 12.5 mg/L ergosterol pre-dissolved in ethanol/Nonidet. As it was proved in a previous work (Carrau et al., 2005), the addition of ergosterol does not affect the levels of isoprenoid biosynthesis. The pH of CDGJM was adjusted to 3.5 with diluted HCl and filter-sterilized. The final concentration (mg/L) of vitamins in CDGJM was *myo*-inositol (100), pyridoxine-HCl (2), nicotinic acid (2), calcium pantothenate (1), thiamine-HCl (0.5), *p*-aminobenzoic acid (0.2), riboflavin (0.2), biotin (0.125), and folic acid (0.2). The final concentration (mg/L) of amino acids in CDGJM was Ala (5.9), Arg (137.3), Asn (36.5), Asp (23.1), Gln (48.7), Glu (30.8), Gly (4.1), His (45.8), Ile (24.1), Lys (61.5), Met (20), Phe (11.6), Ser (48.2), Thr (42.2), Trp (12.1), Tyr (2.4), and Val (24.1). The final concentration (μg/L) of microelements CDGJM was $MnCl_2.4H_20$ (200), $ZnCl_2$ (135), $FeCl_2.4H_2O$ (30), $CuCl_2.2H_2O$ (15), H_3BO_3 (5) $Co(NO_3)_2.6H_2O$ (30), $Na_2MoO_4.2H_2O$ (25) and KIO_3 (10). Leucine and uracil were added as required, at a final concentration of 50 μg/mL. The final YAN was 206 mg N/L. Unless stated otherwise, all chemicals were from Applichem, Germany.

Minimal yeast nitrogen base (YNB at 1.7 g/L) medium was prepared without amino acids ammonium sulfate (Sigma Aldrich, USA), 5 g/L ammonium sulfate, supplemented with 10 g/L glucose, and required amino acids (histidine, leucine) or uracil at 50 μg/mL. The final pH was 4.5.

Yeast strains were routinely propagated at 30 °C in YPD medium. YPD medium is composed of 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose. Yeast strains were stored at −80 °C in 20% glycerol.

A 24-h preculture was prepared from fresh colonies in CDGJM media with selection pressure for plasmid maintenance (leucine or uracil). This preculture was used to inoculate 120 mL of the required filter-sterilized CDGJM medium (in 250 mL Erlenmeyer flasks closed with cotton plugs) to attain an initial concentration of 1×10^5 cells/mL, counted in a Neubauer chamber. Cultures were incubated at 20 °C, with orbital shaking at 150 rpm, in a WIS-30R incubator (Daihan Scientific, South Korea). We have previously confirmed that these conditions avoid the loss of volatiles through the cotton plugs compared to standard cultures at 30 °C and 300 rpm used for more volatile compounds, such as limonene (Jongedijk et al., 2015). At 48 and 72 h (96 h in some experiments evidenced a decrease in the concentration of monoterpenes), cultures were centrifuged to eliminate yeast cells, and the resultant supernatants were adjusted to 50 mg/L SO_2 with potassium metabisulphite and kept at 4 °C until isoprenoid extraction.

Plasmid loss was calculated by replica-plating 100 colonies, first in a minimal medium lacking the amino acids leucine or uracil, depending on the plasmid, and then in a second minimal medium, containing all the required amino acids. Colony growth in the second medium but not in the first indicated plasmid loss.

2.3. Isoprenoid extraction and chemical analysis

The culture media was extracted by solid phase extraction (SPE) using Isolute ENV + ® (Biotage, Sweden). Prior to extraction, SPE cartridges were conditioned, by sequential addition of methanol (MeOH; JT Baker, USA) and ultrapure water (Elga Maxima apparatus, UK). Then, 100 μL of the internal standard 2-octanol (0.352 mg/mL; Sigma–Aldrich, USA) was added to 50 mL of sample, completed to 100 mL, and immediately passed through SPE cartridges. Washing was done with 20 mL ultrapure water and isoprenoids were eluted with 30 mL dichloromethane (CH_2Cl_2; JT Baker, USA). Remaining impurities were removed from cartridges by washing with MeOH (30 mL). Before chemical analysis, the CH_2Cl_2 fraction was concentrated, first with Vigreux type columns, and then by gentle N_2 gas streaming. Biological and technical replicas were performed for all samples.

Isoprenoids were analyzed by gas chromatography–mass spectrometry in a Shimadzu GCMS-QP2010 Ultra equipped with an auto-injector AOC-20i (Shimadzu Co., Japan). The capillary column employed was a DB-Wax (30 m × 0.25 mm × 0.25 μm; Agilent J&W columns, Agilent Technologies, USA). Chromatographic separation was realized under the following conditions: gas carrier, He; flux, 1.38 mL min^{-1}; ion source, 70 eV; injection volume, 1 μL; split ratio, 1:25, and injector, interface, and ion source temperatures were kept at 250 °C. The oven temperature conditions (holding time) were as follows: 40 °C (8 min), increased at 3 °C/min to 180 °C (0 min), then ramped at 5 °C/min to 220 °C (20 min), followed by a further increase at 20 °C/min to 230 °C (7 min).

Identification of the main fermentative volatile compounds was achieved by comparison of the mass spectra with commercially available libraries (NIST, Wiley) and comparison with our previous work (Carrau et al., 2005). To confirm identity, linear retention indexes (LRI) were calculated in the same analytical conditions. In case of the volatile isoprenoids (linalool, geraniol, α-terpineol, (*E*)-nerolidol and (*E,E*)-farnesol) were also injected and co-injected pure standards (all from Sigma-Aldrich). GC–MS analyses were performed in SCAN mode (35–500 *m/z*) for identification and in single ion monitoring (SIM) for quantification purposes. The following ions were selected for every analyte: linalool *m/z*: 71 (Q), 93, 121, 136; geraniol *m/z*: 59, 69 (Q), 81, 93, 95; α-terpineol *m/z*: 59, 93 (Q), 121, 136; (*E*)-nerolidol *m/z*: 69 (Q), 81, 93, 95; (*E,E*)-farnesol *m/z*: 59, 69 (Q), 81, 93. In all cases, Q represents the quantifier ion.

2.4. Statistical analysis

Statistically significant differences for isoprenoids (Figs. 2, 3, and 4) were determined by analysis of variance (ANOVA), using Statistica 7.1 (version 1984–2005; StatSoft, Inc., USA) or post hoc multiple comparison with a least significant difference method in the ANOVA analysis (Fig. 4).

3. Results

In this work, while studying overexpression of the mitochondrial gene *COQ1* that synthesizes the hexaprenyl pyrophosphate synthase, and its effect in isoprenoids formation (Fig. 1), an increase in isoprenoids synthesis was observed, depending, also, on other factors.

3.1. An empty plasmid in a chemically defined medium resembling grape juice results in overproduction of volatile isoprenoids

In order to compare the levels of isoprenoids produced by BY4743 carrying a plasmid versus BY4743, both strains were cultivated in the same CDGJM medium. Pre-culture was performed with plasmid selection (CDGJM without Leu or Ura, depending on the plasmid), but the

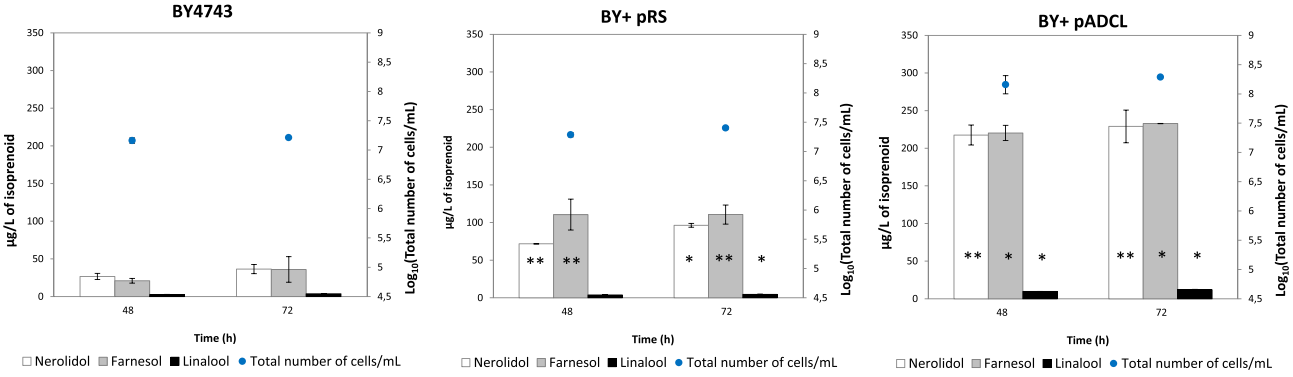


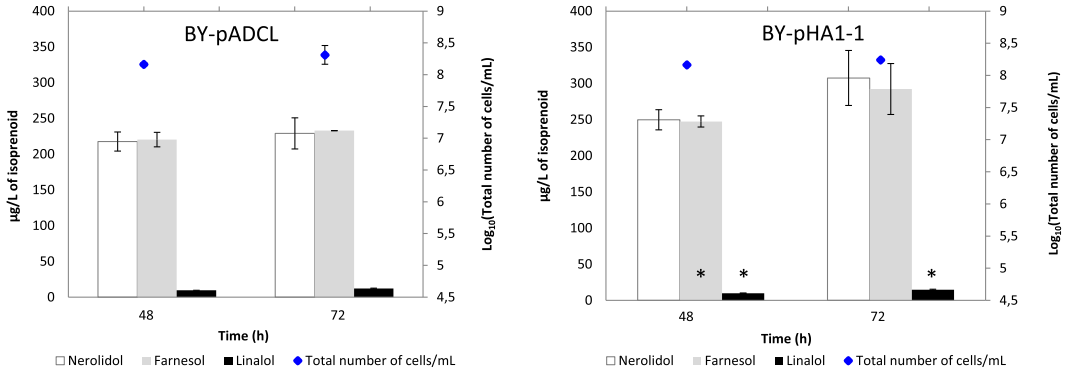
Fig. 2. Levels of linalool, (*E*)-nerolidol, and (*E,E*)-farnesol, and total cell population in BY4743, BY4743 + pRS, and BY4743+ pADCL in chemically defined grape juice medium (CDGJM), containing uracil and leucine. Standard deviations of technical and biological replicates. *, ** indicate significant differences at $p < 0.05$ and $p < 0.01$, respectively.

fermentation media used did not have selection pressure (CDGJM with all the amino acids). At 72 h, the presence of a high-copy-number plasmid (pADCL) versus no plasmid in CDGJM media resulted in significant increases for linalool (3.0-fold; from 3.6 to 12 µg/L), (*E,E*)-farnesol (6.5-fold; from 36 to 232.8 µg/L), and (*E*)-nerolidol (6.3-fold; from 36.5 to 229 µg/L) (Fig. 2). Plasmid loss at 72 h culture without selection pressure was 14%. The total cell population for each treatment was determined, as indicated in the figure, to detect if the changes in growth might affect isoprenoid levels. As a result, the presence of the pADCL plasmid significantly favored the yeast's growth compared to the strain with no plasmid (BY4743) and the strain expressing the low-copy-number plasmid (BY + pRS). No linear correlation could be established between cell growth and isoprenoids formation in Fig. 2. The increase on yeast growth resulted in an increase of total isoprenoid formation, however, when comparing the isoprenoid production with respect to the cell number, the treatment BY + pRS presented the highest values of µg/L·cell^{-1} of (*E,E*)-nerolidol and farnesol compared to BY4743 and BY + pADCL (an average of two to three fold increase production per cell $p < 0,01$, data not shown). However, in the case of the production of linalool, BY4743and BY + pRS presented the highest levels of µg/L·cell^{-1} compared to BY + pADCL (an average increase of three fold production per cell, $p < 0,05$),

When the same experiment was performed in a standard minimal media (YNB), levels of isoprenoids were low, and no significant differences between BY4743 and BY4743 pADCL were found. At 72 h culture, plasmid loss in YNB media was 16%. From these results, it was concluded that the increase in isoprenoid production associated with the presence of plasmids depends on the media composition.

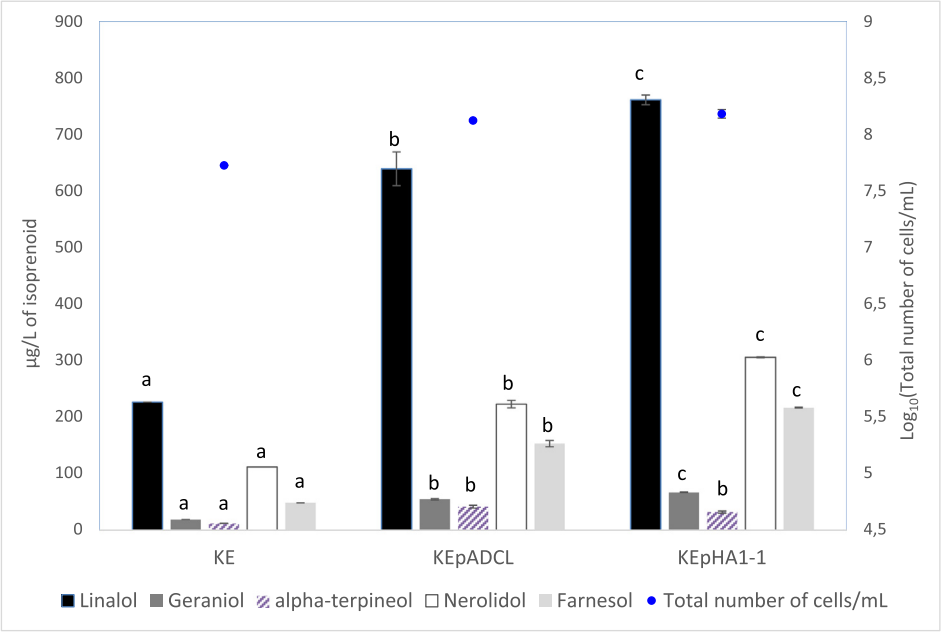
3.2. *COQ1* overexpression from plasmids affects linalool levels

Analyzing BY4743 pHA1–1 (*COQ1* cloned under the constitutive promoter *ADH1* in the high-copy-number plasmid pADCL) versus the same strain with pADCL vector, the levels of linalool detected were significantly higher when *COQ1* was overexpressed. Concentrations of (*E*)-nerolidol and (*E,E*)-farnesol were also higher, although the differences were not statistically significant in all instances (Fig. 3). However, when analyzing the variation of isoprenoids levels per cell number (µg/L·cell^{-1}), the three compounds had a consistent similar increase comparing the strain with the empty plasmid and the strain carrying the plasmid overexpressing *COQ1* being these values of 1,5 folds for (*E,E*) nerolidol, 1,4 for (*E*) farnesol and 1,3 for linalool (bottom table in



isoprenoids per cell (µg/L.cell^{-1})x 10^{-6}	*BY-pADCL (48 h)*	*BY-pADCL (72 h)*	*BY-pHA1-1 (48 h)*	*BY-pHA1-1 (72 h)*
nerolidol	1,45 ab	1,18 a	1,71 b	1,77 b
farnesol	1,46 ab	1,20 a	1,69 b	1,68 b
linalool	0,064 a	0,062 a	0,068 ab	0,084 b

Fig. 3. Levels of linalool, (*E*)-nerolidol, and (*E,E*)-farnesol, and total cell population in BY4743 pADCL and BY4743 pHA1–1 cultivated in chemically defined grape juice medium (CDGJM) with uracil, but without leucine for plasmid selection pressure. Isoprenoid production with respect to cell number are presented in the bottom table for each compound at µg/L·cell^{-1}. Standard deviations of technical and biological replicates. * indicate significant difference at $p < 0.05$. Different letters indicate statistically significant differences at $p < 0.01$.



isoprenoids per cell (µg/L.cell⁻¹)x 10⁻⁶	***KE***	***KEpADCL***	***KEpHA1-1***
linalool	4,2 a	4,81 b	5,0 c
geraniol	0,35 a	0,41 b	0,44 c
nerolidol	2,1 b	1,68 a	2,0 b
farnesol	0,91 a	1,15 b	1,42 c

Fig. 4. Levels of linalool, geraniol, α-terpineol, (*E*)-nerolidol, and (*E,E*)-farnesol produced by strains K197E (KE), K197E pADCL (KE pADCL), and K197E pHA1–1 (KE pHA1–1) in chemically defined grape juice medium (CDGJM), containing uracil and leucine after fermentation for 72 h. Isoprenoid production with respect to cell number are presented in the bottom table for each compound at µg/L·cell^{-1}. Standard deviations of technical and biological replicates. Different letters indicate statistically significant differences at $p < 0.01$.

Fig. 3).

When analyzing BY4743 pRSQ1–1 (*COQ1* cloned under the native promoter from the low-copy-number plasmid pRS316), a statistically significant 1.7-fold increase in the linalool production occurred at 48 h of fermentation, by the same strain carrying an empty vector. For the sesquiterpenes, (*E*)- nerolidol and (*E,E*)-farnesol, even if not statistically significant, an increase of 1.1- and 1.3-fold, respectively, was recorded compared to the strain carrying the empty vector (data not shown).

3.3. Highest isoprenoid levels are obtained when COQ1 is overexpressed in an erg20 mutant context

It has been reported that in an *erg20* mutant context, the production of monoterpenes is exacerbated, probably due to the availability of free GPP (Fischer et al., 2011). Working with an *erg20* mutant, where the amino acid Lys (K) at position 197 was substituted by a Glu (E) (K197E) in a BY4743 genetic context (Fischer et al., 2011), the presence of an empty plasmid (pADCL) increased the levels of isoprenoids (Fig. 4). This increase was even higher if the plasmid overexpressed Coq1p (pHA1–1).

As expected, when an *erg20* mutant was tested, the levels of the monoterpenes, geraniol and linalool, increased almost 100-fold compared to the wild-type BY4743 genetic context, at 72 h of fermentation. Linalool levels in BY4743 were around 2 µg/L while in the K197E background, linalool production was above 200 µg/L, higher than the 78 µg/L reported by Fischer et al. (2011) using a YNB medium. When analyzing the levels of linalool per cell unit, there exist an increase of 19 folds of µg/L·cell^{-1} between BY4743 of Fig. 2 ($0{,}22 \times 10^{-6}$), and the *erg20* mutant strain K197E ($4{,}3 \times 10^{-6}$). In the bottom table of Fig. 4 is presented the production per cell unit of linalool, geraniol, (*E,E*)-nerolidol and farnesol, where a consistent increase of isoprenoid production is seen with KEpADCL and KEpHA1–1 treatments compared to KE (except for (*E,E*)-nerolidol). These data confirm that the increase per cell production could be attributed to plasmids expression and not only to growth. The isoprenoid α-terpineol was excluded from these analyses as it is known to derive by chemical transformations from linalool (Williams et al., 1982; Carrau et al., 2016).

As mentioned before, when KEpHA1.1 (*erg20* mutant strain K197E transformed with a plasmid overexpressing Coq1p) was cultivated in a chemically defined medium that mimics grape juice, the levels of linalool increased reaching 761 µg/L (20% above the empty plasmid). Furthermore, in the K197E genetic context, as shown in Fig. 4, geraniol-related monoterpene was affected in a similar way to linalool by *COQ1* overexpression, with a significant average increase of 22%, respectively, compared to the mutant containing the empty plasmid. These results are also in agreement with our proposed pathways of Fig. 1. However, further studies will be needed to understand the significant average increase of 36–40% found for both sesquiterpenes with *COQ1* overexpression.

4. Discussion

The fact that the presence of an empty plasmid results in overproduction of volatile isoprenoids may be due to the physiological burden that this high-copy-number plasmid imposes on the cells when cultivated in this CDGJM medium, which has a high osmolarity (0.66 M) and low pH (3.5) and YAN level. In consensus with this notion, Karim et al. (2013) studied the effect of plasmid burden on *S. cerevisiae* cells and concluded that different auxotrophies for plasmid selection have different effects on cell physiology. However, their measurements were all performed in YNB medium, and they did not measure isoprenoid production. Ignea et al. (2014) reported high levels of farnesol and nerolidol when genetically modified *S. cerevisiae* cells that overproduce FPP were tested with an empty pYES2 vector in a complete minimal medium. The authors considered that farnesol could be formed via hydrolysis of excess FPP by intracellular phosphatases while nerolidol could be the product of acid hydrolysis of FPP released from the cells. Furthermore, the comparatively higher concentration of YAN in the YNB medium and the presence of metal ions in the CDGJM medium (Mg^{2+}, Fe^{2+}, Mn^{2+}), or both factors, could be some of the important differences between these two fermentation mediums. It is known that these facts could affect isoprenoid formation (Carrau et al., 2005; Huang et al., 2013). On the other hand, growth increase isoprenoids formation but we could not established a linear correlation. However, when production by cell analyses were done, a consistent increase production of isoprenoids per transformed cell was exhibited as is shown in Figs. 3 and 4. Conversely, pADCL plasmid might contribute to the increased leucine concentration in the medium, through its expression of *LEU2* (Hsu and Kohlhaw, 1982). The putative existence of a 3-methylcrotonyl-coenzyme A carboxylase (high scoring sequences with *HFA1*) pathway in *Saccharomyces* (Carrau et al., 2005) might increase the formation of the isoprenoid precursor IPP from leucine at the mitochondrion (Fig. 1). Further studies about these topics should be done to understand this situation and the potential contribution of the leucine catabolism pathway that was only proven with *Aspergillus* strains (Rodrıguez et al., 2004).

Alternatively, the fact that overexpression of *COQ1* by plasmids affects linalool levels suggests it might be interacting mainly in the geraniol–linalool formation, as proposed in Fig. 1 (Carrau et al., 2005). This hypothesis could also concur with the work of Huang et al. (2011, 2013), where, by the generation of different mutants (including a coq1 disruption mutant), these authors proved that coq1 has an important role in GPP formation.

As shown here, higher isoprenoid levels are reached with the pHA1–1 high-copy-number plasmid than those detected with the pRSQ1–1 plasmid, concomitantly with higher levels of Coq1p, as demonstrated previously by Gin and Clarke (2005). As discussed above, there is a known influence of the media composition that supports this increase. However, as stated in the previous paragraph, there is prior evidence that could substantiate our assertion that *COQ1* would be involved in geraniol–linalool formation, which could also be a reason for the increase in the isoprenoid production of the *COQ1* overexpressing strains.

It was shown that isoprenoid production peaked at 72 h of culture and then declined. This trend also corroborates what has been reported for the production of monoterpenes by the fungi *Ceratocystis moniliformis* (Lanza and Palmer, 1977). The 761 μg/L linalool content attained in this study is, to the best of the authors' knowledge, the highest produced by level *S. cerevisiae*, up to now. This concentration is significantly higher than those obtained by several earlier studies that expressed different plant linalool synthases in *S. cerevisiae*, with maximum yields of 95 μg/L (Amiri et al., 2016), 140 μg/L (Rico et al., 2010), 141 μg/L (Pardo et al., 2015), 240 μg/L (Deng et al., 2016), and 313 μg/L (Fischer et al., 2011). The phosphate and pyrophosphate balances during this process might be involved, as indicated in Fig. 1, where Coq1p may be demanding or supplying pyrophosphate to the pathway. Further research of the determination of phosphorylated monoterpenes and sesquiterpenes is required to approach a definitive conclusion to the questions posed in this work.

5. Conclusions

A well-known *S. cerevisiae* laboratory strain produced volatile monoterpenes and sesquiterpenes, depending on several factors, such as medium composition, genetic context, presence of a plasmid, overexpression of native *COQ1*, and the timing of the production peak. The CDGJM, with lower pH (3.5) and lower YAN level compared to standard media, exhibited the highest reported production of linalool and sesquiterpenes by *S. cerevisiae* to date, with positive odor-active values for these compounds. We also demonstrate that it is possible to modulate the levels of isoprenoids *S. cerevisiae* can produce in the absence of plant genes when a model grape juice medium is used. Further studies in model mediums and under real winemaking conditions should be undertaken to understand better the nutrient factors that improve isoprenoid synthesis by *S. cerevisiae*.

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