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Analysis of terpenoid biotransformation in beer by commercial *Saccharomyces cerevisiae* yeast using headspace SPME-GC/MS

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ABSTRACT

Terpenoids are a class of compounds found in hops which are responsible for the distinctive hop aromas in beer. Changes in terpenoid composition during fermentation as a result of yeast biotransformation influences beer aroma, but the pathways involved in these reactions are yet to be fully understood. In this study, eleven terpenoid standards (geraniol, nerol, citronellol, citral, α-terpineol, citronellyl acetate, caryophyllene, linalool, limonene, β-pinene and myrcene) were individually added to a model beer system fermented with commercially available *Saccharomyces cerevisiae* yeast (SafAle US-05). Volatile organic compounds (VOCs) were measured before and after fermentation with headspace solid-phase microextraction gas chromatography/mass spectrometry (SPME-GC/MS). Upon adding a single terpenoid standard to the model beer system, multiple terpenoid products were detected in the resulting beer at the end of fermentation. For example, when geraniol (10 ppm) was added, geraniol, citronellol, citronellyl acetate, citronellal, nerol, dihydrolinalool and dihydrocitronellol were detected at the end of fermentation. This research illustrates the importance of studying individual terpenoids as it provides valuable insights into the complex chemistry of beer. This information can aid in the optimisation of brewing to enhance the production of the flavours and aromas in beer desired by consumers.

1. Introduction

The valuable components in hops (*Humulus lupulus*) for brewing are the bitter acids found in the resin and odour-active compounds found in the essential oils (Stevens, 1967; Verzele et al., 1989; Eyres & Dufour, 2009). Compounds in the hop essential oil are volatile and make an important contribution to the hoppy flavour and aroma of beer, which can be difficult to accurately describe due to it being a complex mixture of over a thousand different volatile compounds (Almaguer et al., 2014).

Terpenoids are a class of VOCs that are responsible for many of these distinctive hop aromas in beer. To meet consumer demand for hop-aroma driven beers, there is increasing interest in controlling, optimising and predicting hop aroma. Few aroma compounds present in hops directly contribute to beer flavour due to changes during fermentation (Sharp et al., 2017). This phenomenon, known as biotransformation, involves the alteration of hop compounds, such as terpenoids, by yeast during fermentation, impacting on the flavour and aroma of the finished beer (Kumar et al., 2023; Richter et al., 2018).

While some biotransformations of terpenoids, like the conversion of geraniol to citronellol, geraniol acetate, and citronellyl acetate, have been observed (King & Dickinson, 2000, 2003; Praet et al., 2012), our understanding is still limited. This limitation primarily stems from the complex composition of hop essential oils, which poses challenges in untangling the formation pathways of specific terpenoid transformations. This complexity is further exacerbated by variations in the essential oils composition between different cultivars, as well as the wide array of outcomes possible with different yeast strains and fermentation conditions (Buiatti et al., 2023; Sharp et al., 2017).

A review by Buiatti et al. (2023) explained the role of various yeast strains in the biotransformation of terpenoids and their potential to impact beer aroma. The review highlighted the complexity of the processes that can occur throughout fermentation and underscores the need for further research to deepen the current understanding. Buiatti et al. (2023) noted that the composition of hop oils and the timing of their addition can significantly alter the aroma that develops during fermentation. Also, that the yeast strain and its enzymatic activity could

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influence terpenoids, thiols, higher alcohols and esters produced (Buiatti et al., 2023). A review by Svedlund et al. (2022) showed the liberation of thiols from cysteine or glutathione-bound adducts, as well as the release of glycosidically bound terpene alcohols and explained how this could increase fruit and floral aromas. These reviews illustrate how aroma formation can be linked to both the biochemical properties of the hops and the functional characteristics of the yeast. A challenge with using whole hops to investigate aroma formation is that it is not possible to differentiate the terpenoid conversion from one compound to the other and the formation due to the liberation from a terpenoid glycoside (Buiatti et al., 2023; Daenen et al., 2008; Svedlund et al., 2022). This makes it difficult to accurately predict how hop additions will impact the aroma of finished beer. Therefore, the objective of the current study was to broaden the understanding of the biotransformation of a wide range of terpenoids by yeast (*Saccharomyces cerevisiae*). Specifically, individual terpenoids such as linalool, geraniol, α-terpineol, citral, citronellyl acetate, citronellol, citronellal, limonene, β-pinene, nerol, β-caryophyllene and myrcene. Each of these terpenoids were individually added into a model beer system (model wort and yeast) and the resultant VOCs produced after fermentation (5 days at 20 °C) were measured using Headspace Solid-Phase Microextraction Gas Chromatography Mass Spectrometry (HS SPME-GC/MS).

2. Materials and methods

2.1. Materials and chemicals

Spray dried malt extract (Briess Golden light) was purchased from a local supplier (BrewShop, Hamilton, NZ). Dried yeast *(S. cerevisiae)*, SafAle US-05 was obtained from Fermentis (Lille, France). Deionized water (18 megaohm·cm) was produced from a Milli-Q Element system (Millipore, Bedford, MA). Iso-α-acids (ICS - I4 Iso Standard) were obtained from American Society of Brewing Chemists (ASBC; Minnesota, USA). Analytical-grade calcium chloride and HPLC-grade ethanol was obtained from Merck Group (Darmstadt, Germany). Instrument grade liquid nitrogen and helium carrier gas (>99.99 %) were obtained from BOC Ltd. (Auckland, NZ). Analytic standards; linalool (Aldrich; ≥97 %), geraniol (Aldrich; ≥99 %), α-terpineol (Supelco; ≥90 %), citral (Sigma-Aldrich; ≥95 %), citronellyl acetate (Sigma-Aldrich; ≥95 %), (*R*)-(+)-limonene (Sigma-Aldrich; ≥97 %), (±)-citronellal (Sigma-Aldrich; ≥95 %), myrcene (Sigma-Aldrich; ≥90 %), ß-pinene (Supelco ≥98.5 %) and GC grade standards; nerol (Fluka; ≥90 %), caryophyllene (Fluka; ≥90 %) and (±)-ß-citronellol (Fluka; ≥90 %) were purchased from Merck Group and were the purest form available (Darmstadt, Germany).

2.2. Model wort preparation and yeast hydration

The model wort was prepared by adding 260 g of malt extract to 1.5 L of milli-Q water. Calcium chloride (50 ppm) was added to enhance wort stability (Merck, Hessen, Germany). Iso-α-acids were also added (ICS - I4 Iso Standard) to obtain 20 International Bitterness Units (IBU). The wort was held at 90 °C for 10 min in a water bath before the temperature was decreased to 20 °C using an ice bath and the gravity was adjusted to 12°P using milli-Q water. The pH of the wort was measured to be 5.2 using a calibrated pH meter. To obtain a target pitching rate of 1×10^7 cells per mL, which was in line with manufacturing recommendations and best brewing practices, 0.5 g of SafAle US-05 (*S. cerevisiae*) yeast was added to 100 mL of cooled wort (12°P, pH 5.2). The sample were then incubated on a shaker (100 rpm) at 20 °C for 30 min.

2.3. Terpenoid dilution

Serial dilutions of each pure terpenoid compound (geraniol, nerol, citronellol, citral, α-terpineol, citronellyl acetate, caryophyllene, linalool, limonene, ß-pinene and myrcene) was made by pipetting 20 μL of the terpenoid and adding it to 1980 μL of ethanol ($dilution_1$). The second dilution was produced by pipetting 20 μL of $dilution_1$ and adding it to 3980 μL of model wort ($dilution_2$). The final dilution that obtained an initial terpenoid concentration of 10 ppm occurred at sample preparation when 100 μL of $dilution_2$ was added to a total of 5 mL.

2.4. Sample preparation and fermentation

Each 5 mL micro-fermentation was prepared in triplicate, adding 3.9 mL of model wort, 1 mL of hydrated yeast, and 100 μL of the terpenoid stock solution to a 20 mL glass headspace vial. Blank controls included samples without terpenoids, which were comprised of 4 mL of model wort and 1 mL of hydrated yeast, and samples without yeast, which contained 4.9 mL of model wort and 100 μL of the terpenoid stock solution. The headspace of each vial was flushed with nitrogen gas at a flow rate of 400 mL/min for 30 s to establish an anaerobic environment. These vials were then sealed and placed into an incubator set at a constant temperature of 20 °C and agitated at 100 rpm for a fermentation duration of 5 days.

2.5. Solid-phase microextraction gas chromatography mass spectrometry (SPME-GC/MS)

SPME-GC/MS was used to isolate, separate and identify the VOCs after the 5-day fermentation. Samples were randomised and placed in a 8 °C Peltier-cooled 32-vial tray attached to a PAL system multipurpose sampler (Zwigen, Switzerland) supplied by Gerstel (Mülheim, Germany). A grey/plain StableFlex SS 1 cm 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) SPME fibre (Merck Group, Darmstadt, Germany) was used to isolate the volatiles from the headspace. Samples were equilibrated for 5 min at 40 °C followed by SPME extraction at 40 °C for 45 min. The SPME fibre was desorbed at 240 °C for 2 min in splitless mode followed by a further 3 min with a purge flow of 50 mL/min.

Gas chromatography was performed with an Agilent Technologies 7890 B (G3440B) gas chromatograph (Agilent Technologies, Beijing, China) coupled to an Agilent Technologies 5977A MSD (Wilmington, DE, USA). Hydrogen gas was used as a gas carrier with a constant flow rate of 1.6 mL/min. Volatiles were separated using a SGE solgel-wax (Trajan, Victoria, Australia) 30 $m \times 0.25$ mm inner diameter $\times$ 0.25 μm film thickness analytical column. The initial oven temperature was 50 °C, then held for 5 min followed by heating at a rate of 5 °C/min to 210 °C, followed by 10 °C/min until 240 °C was reached and held for 5 min. Mass ions were measured between 30 and 300 m/z via (EI mode 70 eV). The ion source temperature was set at 230 °C with a quadrupole temperature of 150 °C. Eluted peaks were identified using NIST 14, RI and authentic standards. The concentration of the eluted peaks was estimated (in ppm) using the calibration equation of the corresponding pure terpenoid standard concentration curves.

2.6. Concentration curve

A concentration curve was generated using serial dilutions (0.3, 0.6, 1.25, 2.5, 5 and 10 ppm) of each pure terpenoid compound in a sodium citrate buffer (0.1 M, pH 5) and ethanol solution. All samples were prepared in triplicate and measured using the SPME-GC/MS method described above. The linearity was satisfactory, with $R^2 > 0.99$ for each compound.

2.7. Refractive index measurement

The degree of fermentation was estimated by measuring the refractive index of the ferment using a handheld refractometer (0–32°Brix) (Fisher Scientific, New Jersey, USA), with measurements taken in triplicate. The °Brix measurement represents the sugar content of an aqueous solution, where one degree Brix corresponds to one gram of sucrose in 100 gs of solution. This is crucial for indicating the solution's

specific gravity, which is essential for determining fermentation progress. The refractometer was calibrated using milli-Q water. For the analysis, 2–3 drops of the samples after fermentation were pipetted onto the refractometer's sample plate, and the cover plate was gently closed. After allowing 30 s for the sample to spread, any dry spots or bubbles were corrected by adding an additional drop. The refractometer was then raised to eye level and pointed toward a light source for observation. The °Brix measurement was recorded. After use, the refractometer was cleaned with milli-Q water and dried with a microfiber cloth. To correct for the alcohol's effect on the refractive index, the specific gravity was calculated using Eq (1).

$$Specific\ gravity = \left(\frac{Brix}{258.6 - \left(\left(\frac{Brix}{258.2} \right) \times 227.1 \right)} \right) + 1 \quad (1)$$

2.8. Data analysis

The raw SPME-GC/MS data was exported from the Agilent MassHunter software (Version B.07.02.1938, Agilent Technologies, Beijing, China) and processed using PARAFAC2 based Deconvolution and Identification System (PARADISe) software (version 3.9). PARADISe was used to obtain the compound identities and relative abundances by converting the raw data in the form of a netCDF data file into a peak table (Johnsen, Skou, Khakimov, & Bro, 2017; Warburton, Silcock, & Eyres, 2022). PARADISe can differentiate overlapping signals to produce low signal-to-noise ratio for peaks of all samples at a given retention time even with a high number of samples (Skov & Bro, 2008). PARADISe also identifies peaks based on deconvoluted mass spectra applying integrated search engine, and greatest peak identification report. The search engine used was National Institute of Standards and Technology (NIST 2014) database. The identity of the volatile compounds was reviewed and confirmed based on comparison to the chemical standards and the library matches (≥80 %) in the NIST Mass Spectral Library.

3. Results

The gravity and pH of each ferment (3 replications) was measured at the start (original) and end (final) of fermentation (Table 1). For samples containing yeast, the pH of the wort after boiling (5.0–5.2) decreased during fermentation to between 4.0–4.4. The gravity also decreased from between 1.048–1.049 to between 1.023–1.024 after fermentation, illustrating that fermentation had occurred. The observed final gravity was higher than expected, which may have been influenced by the volume of the fermentations (5 mL). A smaller volume could have led to earlier yeast sedimentation, pushed by the higher surface-to-volume ratios, causing yeast cells to settle more quickly before fully metabolising all the available sugars. It is still expected that the use of microfermentations will accurately mirror the yeast biotransformations observed in larger-scale fermentations. As expected, the pH and gravity of the samples without yeast did not decrease, confirming that fermentation did not occur and there was no contamination.

Terpenoids were not detected in the fermented model wort to which had not been added (control), which confirmed that *S. cerevisiae* does not indigenously produce terpenoid compounds during fermentation. In addition, in unfermented wort without yeast, only the terpenoids added were detected after a 5-day incubation supporting a previous observation that these terpenoids are unable to spontaneously transform (King & Dickinson, 2000).

In contrast, after fermentation the model wort spiked with a single terpenoid contained a range of terpenoids (Table 2), thereby suggesting that the yeast cells during fermentation were playing a role in the biotransformation of the terpenoids. Table 2 displays the concentration (ppm) of terpenoids at the end of fermentation (5 days at 20 °C).

Table 1
Comparative analysis of original and final gravity and pH in different wort samples (with and without yeast) supplemented with single terpenoids (mean ± standard deviation, $n = 3$).

Sample (Wort plus a single terpenoid with or without yeast)	Original gravity	Original pH	Final gravity	Final pH
Geraniol	1.05 ± 0.00	5.14 ± 0.05	1.02 ± 0.00	4.33 ± 0.05
Nerol	1.05 ± 0.00	5.25 ± 0.07	1.02 ± 0.00	4.22 ± 0.05
Citral	1.05 ± 0.00	5.20 ± 0.01	1.02 ± 0.00	4.41 ± 0.05
α-terpineol	1.05 ± 0.00	5.19 ± 0.05	1.02 ± 0.00	4.27 ± 0.01
Citronellyl acetate	1.05 ± 0.00	5.30 ± 0.01	1.02 ± 0.00	4.27 ± 0.02
Caryophyllene	1.05 ± 0.00	5.24 ± 0.01	1.02 ± 0.00	4.30 ± 0.04
Linalool	1.05 ± 0.00	5.17 ± 0.00	1.02 ± 0.00	4.33 ± 0.11
Limonene	1.05 ± 0.00	5.30 ± 0.03	1.02 ± 0.00	4.33 ± 0.12
β-pinene	1.05 ± 0.00	5.17 ± 0.03	1.02 ± 0.00	4.33 ± 0.13
Citronellol	1.05 ± 0.00	5.18 ± 0.01	1.02 ± 0.00	4.30 ± 0.4
Wort with yeast (control)	1.05 ± 0.00	5.28 ± 0.04	1.02 ± 0.00	4.33 ± 0.14
Wort without yeast (blank)	1.05 ± 0.00	5.28 ± 0.03	1.05± 0.00	5.26 ± 0.025

4. Discussion

4.1. Biotransformation of geraniol

In the model wort spiked with geraniol (10 ppm), the terpenoids detected at the end of fermentation were geraniol, citronellol, citronellyl acetate, nerol, citronellal, dihydrocitronellol and dihydrolinalool (Table 2). Citronellol and citronellyl acetate have previously been identified as products of geraniol biotransformation by yeast in fermentation studies (King & Dickinson, 2000, 2003; Steyer et al., 2013; Takoi et al., 2010).

King and Dickinson (2000) investigated the biotransformation of geraniol (25 ppm) by *Saccharomyces cerevisiae* IWD72, *Kluyveromyces lactis IFO1267* and *Torulaspora delbrueckii NCYC* 696 yeast in minimal medium (containing 2 % glucose, 0.5 % ammonium sulphate, 0.17 % Difco yeast nitrogen base) over 3 days at an unreported temperature and detected different terpenoids when different yeast was used. Geraniol, linalool and α-terpineol was detected after fermentation by *T. delbruecki*. Geraniol, citronellol, linalool and α-terpineol was detected after fermentation by *S. cerevisiae* and by *K. lactis*. King and Dickinson (2003) then investigated the biotransformation of geraniol (10 ppm) by either an ale (*Saccharomyces cerevisiae* NCYC 1681) or a lager (*Saccharomyces bayanus* NCYC 1324) yeast in fermentose-based medium over 15 days. It was reported that citronellol, nerol, linalool, α-terpineol, geranyl acetate and citronellyl acetate were detected in the ale yeast ferments and citronellol, nerol, linalool, α-terpineol, geranyl acetate and citronellyl acetate were detected in the lager yeast (*S. bayanus* NCYC 1324) ferments. King and Dickinson (2003) proposed that geraniol was primarily transformed to citronellol and subsequently to linalool. While linalool was not detected in the current study, dihydrolinalool was, and it is speculated that it could have been formed by the reduction of linalool. While the formation of dihydrolinalool has not previously been reported to occur as a result of yeast fermentation, bacteria such as *Pseudomonas putida* have been shown to transform myrcene into dihydrolinalool, cis-β-dihydroterpineol, and linalool (Esmaeili et al., 2021).

Fermentation of a synthetic fermentation medium and (MS300)

Table 2
Biotransformation of terpenoids by SafAle US-05 (*S. cerevisiae*) yeast. Compounds: geraniol, nerol, citral, α-terpineol, citronellyl acetate, caryophyllene, linalool, limonene, ß-pinene and citronellol were individually spiked at a concentration of 10 ppm. Samples were measured after 5-days incubation at 20 °C using SPME-GC/MS (mean ± standard deviation, $n = 3$).

	Terpenoids detected (ppm)								
Terpenoids added (10 ppm)	Geraniol	Nerol	Citral	Citronellyl acetate	Linalool	ß-pinene	Citronellol	Limonene	Caryophyllene
Geraniol	1.64 ± 0.20	0.09 ± 0.01		0.62 ± 0.01			1.64 ± 0.18		
Nerol	0.22 ± 0.01	3.45 ± 0.87		0.09 ± 0.00			0.65 ± 0.09		
Citral	1.48 ± 0.31	0.77 ± 0.16	0.02 ± 0.01	0.20 ± 0.03			1.85 ± 0.26		
α-terpineol			0.01 ± 0.00			0.11 ± 0.02			
Citronellyl acetate	0.01 ± 0.00	0.02 ± 0.01		0.60 ± 0.46			1.31 ± 1.22		
Caryophyllene		0.01 ± 0.00			0.13 ± 0.03	0.01 ± 0.00			0.65 ± 0.25
Linalool	0.01 ± 0.00	0.01 ± 0.00			2.58 ± 0.35			0.01 ± 0.00	
Limonene		0.04 ± 0.06						0.90 ± 0.24	
ß-pinene	0.01 ± 0.00					1.72 ± 0.06		0.01 ± 0.01	
Citronellol		0.03 ± 0.00		0.45 ± 0.03			3.88 ± 0.02		

	Terpenoids detected (ppm)						
Terpenoids added (10 ppm)	α-terpineol	Dihydrocitronellol	Citronellal	Humulene	Dihydrolinalool	Nerol acetate	Myrcene
Geraniol		0.04 ± 0.00	0.04 ± 0.00		0.02 ± 0.00		
Nerol	0.07 ± 0.01	0.02 ± 0.00			0.02 ± 0.00	0.18 ± 0.02	
Citral		0.04 ± 0.00	0.05 ± 0.01			0.02 ± 0.01	
α-terpineol	3.58 ± 0.24						
Citronellyl acetate					0.03 ± 0.01		
Caryophyllene				0.40 ± 0.14			
Linalool	0.01 ± 0.00		0.02 ± 0.01		0.60 ± 0.28		0.02 ± 0.01
Limonene							
ß-pinene	0.09 ± 0.02						
Citronellol		0.06 ± 0.00		0.04 ± 0.00	0.02 ± 0.00		

spiked with geraniol (1 ppm) by two *S. cerevisiae* wine yeast strains (59a and S288c) resulted in the detection of citronellol, linalool, nerol, geranyl acetate and citronellyl acetate after 25 h (Steyer et al., 2013). Both yeast strains produced the same terpenoids; however, the concentrations of the compounds were strain-dependant as 59a produced a higher concentration of acetates compared to S288c. Steyer et al. (2013) showed that when the yeast strain *S. cerevisiae* BY4741 had the gene for the production of the OYE2 enzyme removed, there was a dramatic decrease in the concentration of citronellol formed from geraniol. When the enzyme was overexpressed, the conversion of geraniol to citronellol increased by 37 % compared to the control/wild strain. (Steyer et al., 2013).

The differences in terpenoids detected after fermentation of geraniol from literature and the current study can be attributed to several factors such as strain- or species-dependent biotransformation reactions. Different yeast strains or species have different enzymatic profiles which could lead to the transformation of geraniol into various terpenoids (Jiang et al., 2023). For instance, *T. delbrueckii* was found to produce geraniol, linalool, and α-terpineol, while S. cerevisiae and *K. lactis* produced an additional compound, citronellol. Additionally, fermentation conditions such as medium composition, temperature, and duration could influence the terpenoids produced by altering the reaction pathways involved (Kumar et al., 2023). Overall, these findings emphasise the intricate nature of geraniol biotransformation during fermentation, underscoring the importance of considering both strain-specific differences and fermentation conditions when studying its biotransformation.

4.2. Biotransformation of nerol

In the model wort spiked with nerol (10 ppm), terpenoids detected at the end of fermentation were nerol, citronellol, geraniol, nerol acetate, citronellyl acetate, α-terpineol, dihydrocitronellol and dihydrolinalool (Table 2).

King and Dickinson (2000) investigated the biotransformation of nerol (25 ppm) by *S. cerevisiae* IWD72, *K. lactis IFO1267* or *T. delbrueckii NCYC* 696 yeast in minimal medium (containing 2 % glucose, 0.5 % ammonium sulphate, 0.17 % Difco yeast nitrogen base) over 3 days at an unreported temperature. It was observed that different terpenoids were produced through the biotransformation of nerol by the three different yeast species. Linalool, α-terpineol and geraniol was detected after fermentation by *T. delbrueckii*. Only α-terpineol was detected after fermentation by *S. cerevisiae*. Linalool, α-terpineol, geraniol and citronellol was detected after fermentation by *K. lactis* (King & Dickinson, 2000). The detection of citronellyl acetate, dihydrocitronellol, dihydrolinalool and nerol acetate from nerol in the current study by yeast has not previously been reported.

4.3. Biotransformation of citronellol

In the model wort spiked with citronellol (10 ppm), the terpenoids detected at the end of fermentation were citronellol, citronellyl acetate, nerol, dihydrocitronellyol, humulene and dihydrolinalool (Table 2).

Our findings align with previous research that identified the acetylation of citronellol to citronellyl acetate in a model system using *S. cerevisiae* AWRI 796 (Slaghenaufi et al., 2020). The acetylation of terpene alcohols has been shown to be strain-dependent and influenced by the activity of Atf1 alcohol acetyltransferase (King & Dickinson, 2003; Rojas et al., 2001; Steyer et al., 2013) The presence of other terpenoids like nerol, dihydrocitronellol, humulene, and dihydrolinalool suggests that multiple biotransformation pathways are active during fermentation. The similar terpenoids detected after the fermentation of geraniol suggest an overlapping transformation pathway. The hypothesised formation pathway is presented in Fig. 1. However, further studies are needed to understand the enzymatic mechanisms behind these biotransformations.

4.4. Biotransformation of citral

In the model wort spiked with citral (10 ppm), terpenoids detected at the end of fermentation were citral, geraniol, nerol, citronellyl acetate, citronellol, dihydrocitronellol, citronellal and nerol acetate (Table 2). A previous study investigated the bioconversion of citral (undefined concentration) in PGE (peptone broth/glucose/yeast extract) by free and immobilized *S. cerevisiae* after 15 days, and reported detection of α-terpineol, limonene, α-pinene, geraniol and citronellol (Esmaeili et al., 2012). The absence of α-terpineol, limonene and α-pinene in the current

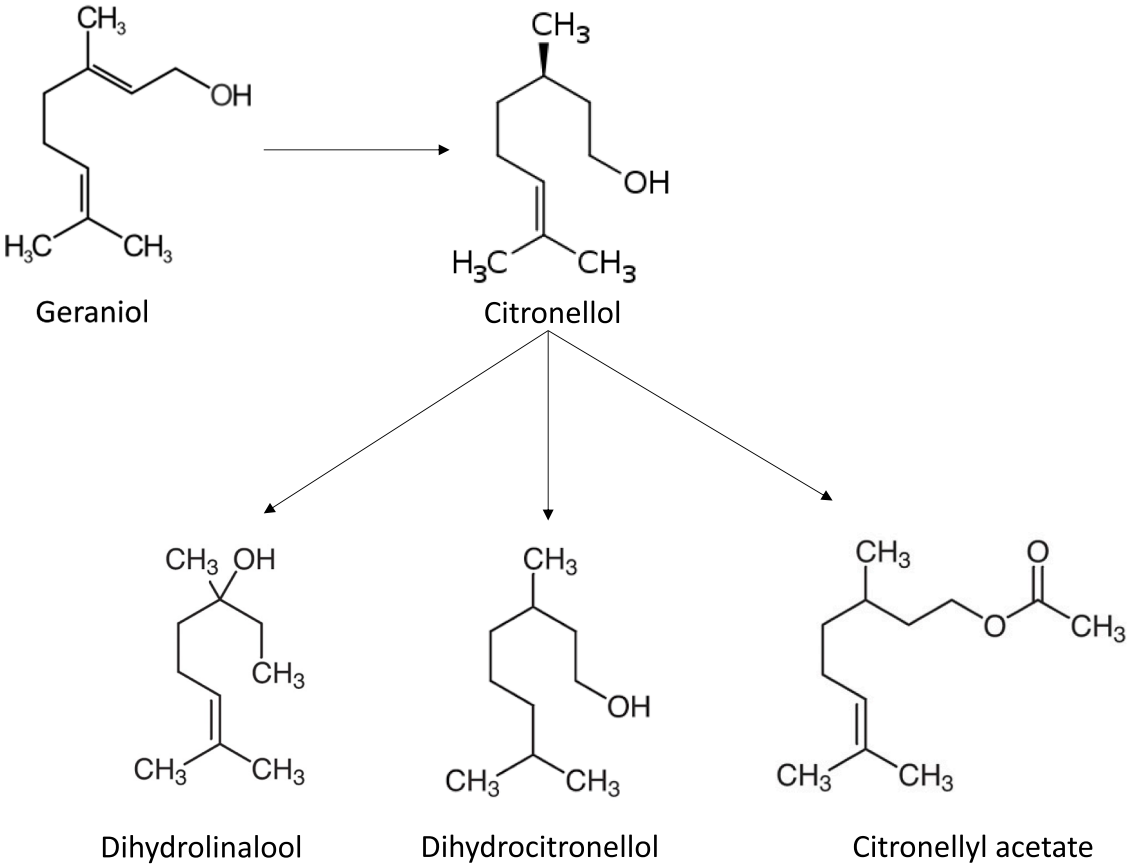

Fig. 1. Proposed biotransformation pathway of geraniol to citronellyl acetate, dihydrocitronellol and dihydrolinalool via citronellol.

study, despite its detection in previous research by Esmaeili et al. (2012), could be attributed to the different fermentation conditions employed (23 °C for 15 days in the previous study vs 20 °C for 5 days in the current study). Additionally, the absence of these terpenoids may be a result of strain-specific differences in enzymatic activity or metabolic pathways, leading to the different biotransformation products.

Mäki-Arvela et al. (1997) investigated the hydrogenation kinetics of citral (5 ppm) in a semi-batch reactor with nickel as a catalyst over 5 h. The kinetic experiments revealed that the conjugated double bond is the most reactive one, yielding citronellal as a primary product. Hydrogenation of the carbonyl group in citronellal gave citronellol as the secondary reaction product. After 5 h, the hydrogenation of citronellol to dihydrocitronellol (3,7-dimethyloctanol) was detected. In parallel, formation of nerol and geraniol was also observed but at lower concentrations. Fig. 2 displays possible reactions and products of citral (Mäki-Arvela et al., 1997).

4.5. Biotransformation of α-terpineol

In the model wort spiked with α-terpineol (10 ppm), terpenoids detected at the end of fermentation were α-terpineol, citral and ß-pinene (Table 2). The detection of ß-pinene and citral from α-terpineol by yeast has not previously been reported and suggests that *S.cerevisiae* SafAle US-05 may possess uncharacterised enzymatic pathways for the biotransformation of α-terpineol, which requires further investigation.

4.6. Biotransformation of citronellyl acetate

In the model wort spiked with citronellyl acetate (10 ppm), terpenoids detected at the end of fermentation were citronellyl acetate, geraniol, nerol, citronellol and dihydrolinalool (Table 2). As previously mentioned, King and Dickinson (2003) detected citronellyl acetate produced from citronellol after fermentation by *S. cerevisiae*. The current study is the first study to report the reverse reaction by *S. cerevisiae*, where citronellyl acetate is produced from citronellol during fermentation. Previous research had shown that citronellyl acetate (10 ppm) was biotransformed by *Aspergillus niger* in potato dextrose agar (PDA) into citronellol and citronellol hydrate (Madyastha & Murthy, 1988).

Geraniol, nerol and dihydrolinalool have not previously been reported as products of citronellyl acetate biotransformation by *S. cerevisiae.* Citronellyl acetate was found to produce nerol, geraniol, and citronellol during biotransformation. Since geraniol has been shown to produce citronellol, it is possible that citronellyl acetate can interconvert to citronellol and geraniol. However, these interconversions might have been missed due to a lack of measurements taken throughout the fermentation process. Additionally, when nerol was spiked into the model wort, citronellyl acetate was produced, indicating that nerol and citronellyl acetate may also be able to interconvert.

4.7. Biotransformation of caryophyllene

In the model wort spiked with caryophyllene (10 ppm), terpenoids detected at the end of fermentation were caryophyllene, nerol, linalool, ß-pinene and humulene (Table 2). King and Dickinson (2003) previously investigated caryophyllene (10 ppm) biotransformation by either *S. cerevisiae* (NCYC 1681) or *S. bayanus* (NCYC 1324) yeast in yeast extract peptone dextrose (YEPD) medium over 15 days and detected no terpenoids (King & Dickinson, 2003). In an experiment investigating caryophyllene (10 ppm) biotransformation by *Wolfiporia extensa* fungus in standard nutrient liquid medium (SNL) over 7 days, humulene was detected (Batur et al., 2019). The disappearance of caryophyllene during beer fermentation has previously been theorised to result from adsorption by yeast cells and migration to the foam layer, with no terpenoids detected as biotransformation products (King & Dickinson, 2003; Praet

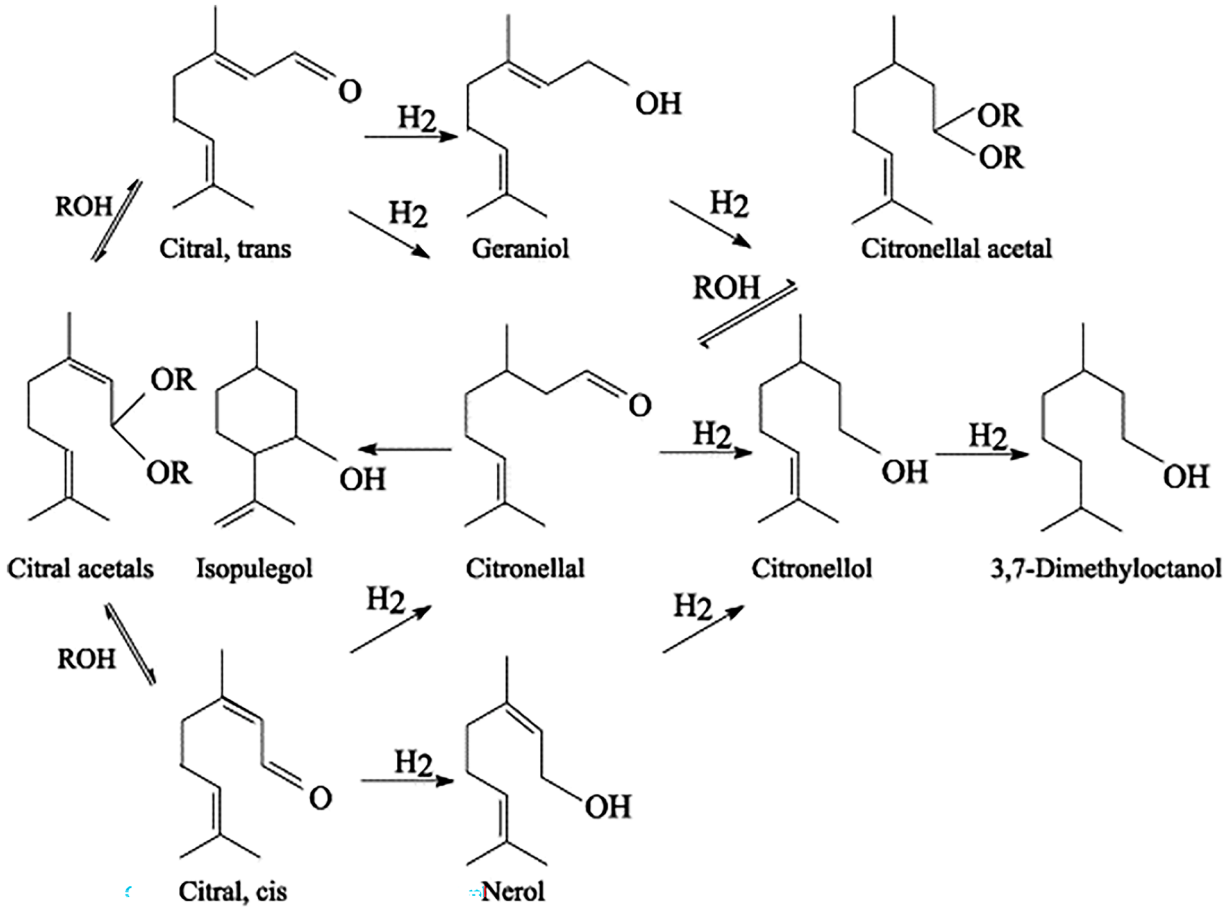

Fig. 2. Possible reactions (cyclisation, acetalisation, decarbonylation, dehydrogenation) of citral (*cis* and trans) (Mäki-Arvela et al., 1997).

et al., 2012; Siebert, 1994). The results of the current study show that the disappearance of caryophyllene could also be attributed to its biotransformation into nerol, linalool, ß-pinene, and humulene.

4.8. Biotransformation of linalool

In the model wort spiked with linalool (10 ppm), terpenoids detected at the end of fermentation were linalool, geraniol, nerol, limonene, α-terpineol, citronellal, dihydrolinalool and myrcene (Table 2).

King and Dickinson (2000) examined the biotransformation of linalool (25 ppm) using yeast strains *S. cerevisiae* (IWD72), *T. delbrueckii* (NCYC 696), and *K. lactis* (IFO1267) in a minimal medium. Over a 3-day period, α-terpineol was identified as a product. Extending their work, King and Dickinson (2003) also explored linalool (10 ppm) biotransformation in fermentose-based medium over 15 days using ale (*S. cerevisiae* NCYC 1681) and lager (*S. bayanus* NCYC 1324) yeast strains, reporting α-terpineol, geraniol, and nerol as fermentation products. Brodkorb et al. (2010) found that *Castellaniella defragrans* (65Phen) bacteria transformed linalool (1.5 ppm) to myrcene and geraniol in Tris–HCl buffer within 6 h (Brodkorb et al., 2010). Mirata et al. (2008) investigated *S. cerevisiae bayanus* strains Zymaflor VL1 and Uvaferm 228 for their ability to biotransform linalool (50 ppm) in MYB medium over 5 days. Both strains produced myrcene, limonene, dihydrolinalool, α-terpineol, and citronellol, with Zymaflor VL1 also yielding geraniol and citral. (Mirata et al., 2008). The detection of ß-pinene and citronellal from linalool by *S. cerevisiae* has not previously been reported.

4.9. Biotransformation of limonene

In the model wort spiked with limonene (10 ppm), the terpenoids detected at the end of fermentation were limonene and nerol (Table 2).

The biotransformation of limonene to nerol by *S. cerevisiae* has not previously been reported. Fig. 3 displays previously identified biotransformations of limonene combined with the results from the current study. It has been observed that nerol can be produced from α-terpineol by *Sphingobium* sp over a period of 2 days. In the past, the biotransformation of limonene to α-terpineol has also been described (Bicas et al., 2010). There have been reports of limonene and nerol being produced through the biotransformation of geraniol (100 ppm) in YMPG liquid medium (consisting of yeast extract, malt extract, bacteriological peptone, and glucose) by *Aspergillus niger* after 7 days at an undefined temperature (Demyttenaere et al., 2000). Additionally, it has been noted that microorganisms can form intermediate compounds, such as linalool, which is an intermediate in the conversion of nerol to α-terpineol by *Aspergillus niger* (Demyttenaere & Willemen, 1998). Based on the current study and previous literature, it is hypothesized that α-terpineol is an intermediate in the biotransformation of limonene to nerol by *S. cerevisiae*.

4.10. Biotransformation of ß-pinene

In the model wort spiked with ß-pinene (10 ppm), the terpenoids detected at the end of fermentation were ß-pinene, geraniol, limonene and α-terpineol (Table 2). ß-pinene is one of the most abundant bicyclic monoterpene hydrocarbons found in plants (Schwab et al., 2013). Soares-Castro et al. (2021) stated that ß-pinene can be used to produce virtually all monoterpenes aroma compounds used in the food industry

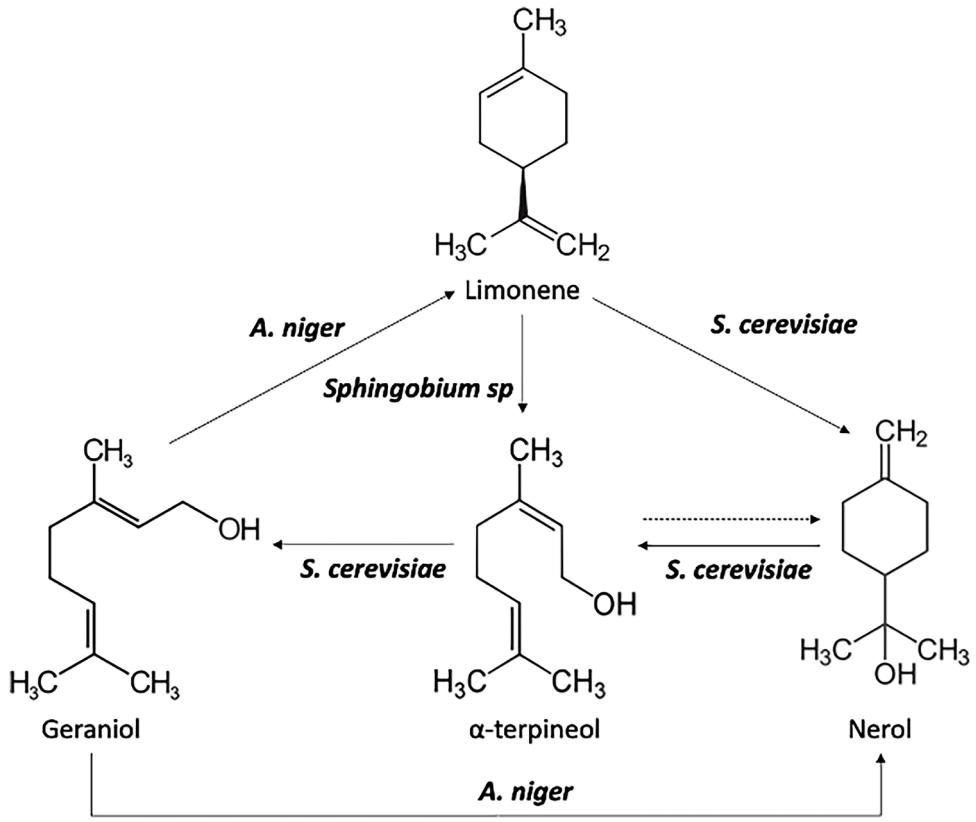

Fig. 3. Potential pathway for the formation of nerol from limonene (limonene to α-terpineol by *Sphingobium sp* was reported by Bicas et al. (2010). Bioconversion of geraniol to limonene and nerol by *Aspergillus niger* was reported by Demyttenaere et al. (2000). The hypothesised intermediate biotransformation from α-terpineol to nerol has been indicated by a dashed arrow.

(Soares-Castro et al., 2021).

A previous study that investigated ß-pinene (10 ppm) biotransformation by *Aspergillus niger* (ATCC 16,404, ATCC 9642 and ATCC 1004) and *Penicillium camembertii* (ATCC 4845) in potato dextrose agar (PDA) medium over 3 days detected α-terpineol from all microorganisms (Rottava et al., 2010). Soares-Castro et al. (2017) investigated the biotransformation of ß-pinene (5 ppm) by *Pseudomonas sp* (M1) in minimal medium for 20 h, and detected limonene and α-terpineol (Soares-Castro, Montenegro-Silva, Heipieper, Santos et al., 2017). Geraniol has not previously been detected as a product of ß-pinene biotransformation by microorganisms.

Fig. 4 (adapted from Soares-Castro et al., 2021) displays the most common metabolic pathways described for biotransformation of ß-pinene by bacteria. Additional terpenoids found to be a product of ß-pinene and α-pinene biotransformation (that are also associated with terpenoids found in beer) are: linalool, citral, ß-citronellol and nerol (Soares-Castro et al., 2021).

4.11. Biotransformation of myrcene

In the model wort spiked with myrcene (10 ppm), no terpenoids were detected at the end of fermentation (Table 2). These results align with literature, as when King and Dickinson (2003) added 10 ppm of ß-myrcene to yeast *S. cerevisiae* NCYC 1681 and *S. bayanus* NCYC 132 no terpenoids were detected (after 15 days of fermentation). King and Dickinson hypothesised that the inability to detect myrcene or any terpenoids was due to evaporation, poor solubility or yeast absorption. It was concluded that the absence of myrcene was irrelevant to beer brewing due to the low concentration of myrcene in finished beer and therefore its very limited impact on beer flavour (King & Dickinson, 2003).

5. Conclusion

Terpenoids play an important role in the development of hop aroma in beer. The ability to better understand and control the formation of terpenoids using yeast would allow brewers to manipulate the sensory profile of beer. Biotransformation of individual terpenoids usually results in the production of multiple products, increasing the complexity of the aroma profile of beer. In a study evaluating the preference of over 1.5 million consumers on the aroma compositions of different beers, it was observed that beers with diverse aroma profiles had a greater acceptance. Specifically, beers that contained a higher concentration of oxygenated terpenoids such as citronellol, linalool, α-terpineol were generally preferred by consumers compared to beers that contained a lower concentration of oxygenated terpenoids (Paiva & Hantao, 2020).

Micro-fermentations have previously been used to track the behaviour of VOCs during the fermentation of beer (Richter et al., 2018). Richter et al. (2018) also reported relatively poor recovery of terpenoids spiked after fermentation, which might indicate a limitation of the analysis method in fully capturing VOCs. This limitation might also explain why samples without yeast exhibited a relatively poor recovery rate, although the decrease was less than in samples where yeast was present. In samples without yeast, a decrease in terpenoid concentration indicated that sampling alone resulted in losses. The recovery in samples with yeast was lower, likely due to the absorption and biotransformation of the spiked terpenoid.

The results of the current study showed that the biotransformation of individual terpenoids increased the aroma profile complexity since multiple terpenoids were detected after fermentation. For example, from nerol (floral, fresh and green aroma), seven terpenoids were detected including citronellol and citronellyl acetate, which would add a citronella oil, rose and fruity aroma to the final beer (if above threshold concentration) (Bauer et al., 2008; Paiva & Hantao, 2020). This study

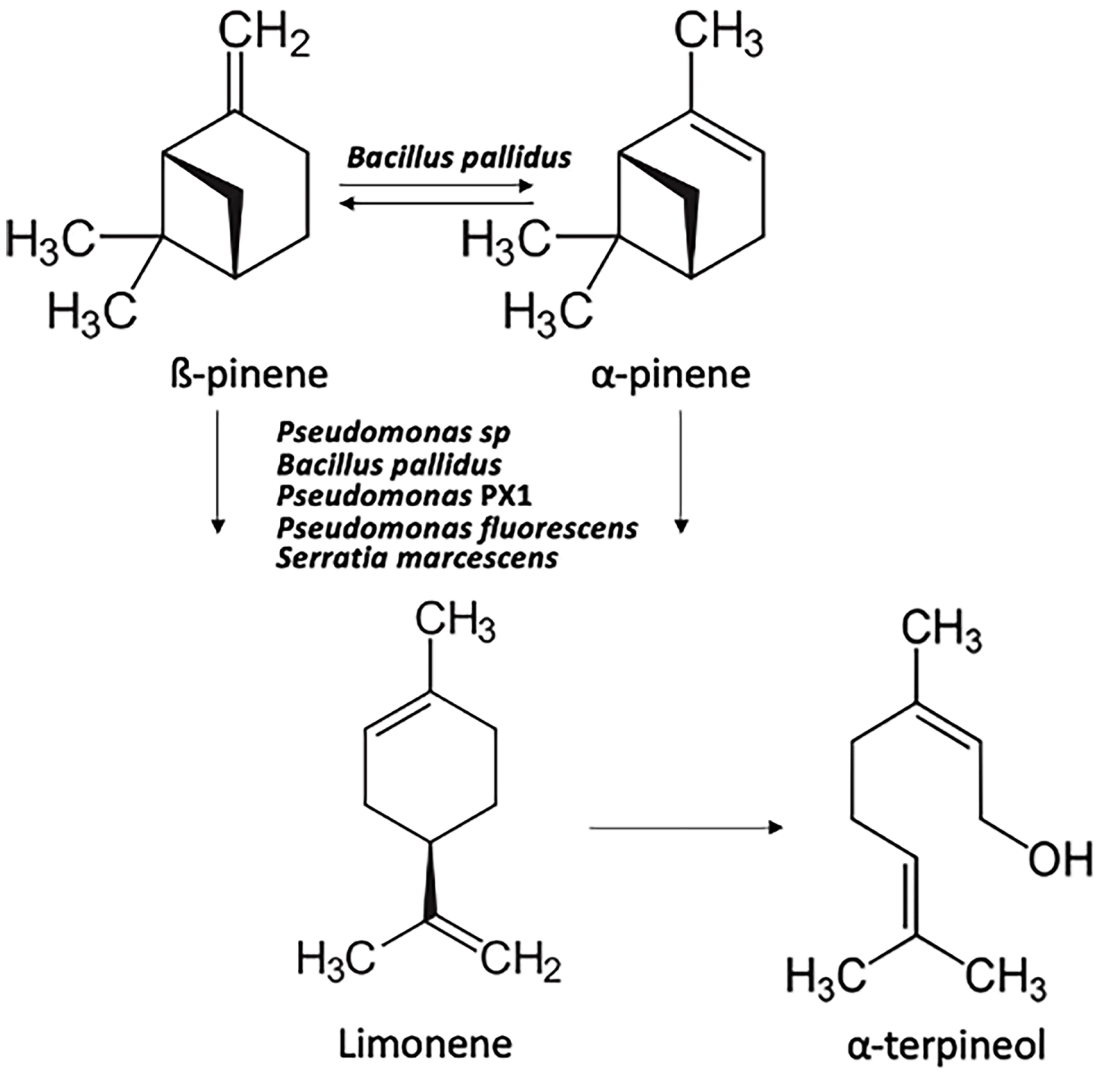

Fig. 4. Most common metabolic pathways of ß-pinene described by bacteria (adapted from Soares-Castro et al., 2021). Isomerization between ß-pinene and α-pinene by *Bacillus pallidus* (Savithiry et al., 1998). ß-pinene and α-pinene to limonene followed by α-terpineol by *Pseudomonas sp, Bacillus pallidus, Pseudomonas PX1, Pseudomonas fluorescen* and *Serratia marcescens* (Cheng et al., 2012; Colocousi et al., 1996; Gibbon & Pirt, 1971; Savithiry et al., 1998; Soares-Castro, Montenegro-Silva, Heipieper & Santos, 2017; Wright et al., 1986; Yoo & Day, 2002).

measured a range of terpenoids as an initial insight into which compounds are undergo biotransformation by yeast. Additionally, this research has identified biotransformation products not previously reported, expanding the current understanding. However, further research is required to monitor the development of the VOCs throughout fermentation to gain an understanding of the formation pathways and development of hop aroma in beer.

CRediT authorship contribution statement

Rebecca Roberts: Writing – original draft, Investigation, Formal analysis. **Patrick Silcock:** Writing – review & editing, Supervision, Conceptualization, Methodology. **Michelle Leus:** Formal analysis. **Franco Biasioli:** Writing – review & editing, Supervision, Conceptualization. **Phil Bremer:** Writing – review & editing, Supervision, Conceptualization, Methodology. **Graham T. Eyres:** Writing – review & editing, Supervision, Conceptualization, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Rebecca Roberts reports a relationship with University of Otago and Fondazione Edmund Mach that includes: funding grants- doctoral scholarship.

Data availability

Data will be made available on request.

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References

Almaguer, C., Schönberger, C., Gastl, M., Arendt, E. K., & Becker, T. (2014). *Humulus lupulus* – a story that begs to be told. A review. *Journal of the Institute of Brewing, 120* (4), 289–314. https://doi.org/10.1002/jib.160

Batur, Ö.Ö., Kiran, İ., Berger, R. G., & Demirci, B. (2019). Microbial transformation of β-caryophyllene and longifolene by Wolfiporia extensa. *Natural Volatiles and Essential Oils, 6*(3), 8–15.

Bauer, K., Garbe, D., & Surburg, H. (2008). *Common fragrance and flavor materials: Preparation, properties and uses*. John Wiley & Sons.

Bicas, J. L., Fontanille, P., Pastore, G. M., & Larroche, C. (2010). A bioprocess for the production of high concentrations of R-(+)-α-terpineol from R-(+)-limonene. *Process Biochemistry, 45*(4), 481–486. https://doi.org/10.1016/j.procbio.2009.11.007

Brodkorb, D., Gottschall, M., Marmulla, R., Lüddeke, F., & Harder, J. (2010). Linalool dehydratase-isomerase, a bifunctional enzyme in the anaerobic degradation of monoterpenes. *Journal of Biological Chemistry, 285*(40), 30436–30442.

Buiatti, S., Tat, L., Natolino, A., & Passaghe, P. (2023). Biotransformations performed by yeasts on aromatic compounds provided by hop—A review. *Fermentation, 9*(4), 327.

Cheng, Z., Sun, P., Jiang, Y., Zhang, L., & Chen, J. (2012). Kinetic analysis and bacterium metabolization of α-pinene by a novel identified *Pseudomonas* sp. strain. *Journal of Environmental Sciences, 24*(10), 1806–1815.

Colocousi, A., Saqib, K., & Leak, D. (1996). Mutants of Pseudomonas fluorescens NCIMB 11671 defective in the catabolism of α-pinene. *Applied Microbiology And Biotechnology, 45*(6), 822–830.

Daenen, L., Saison, D., Sterckx, F., Delvaux, F. R., Verachtert, H., & Derdelinckx, G. (2008). Screening and evaluation of the glucoside hydrolase activity in *Saccharomyces* and *Brettanomyces* brewing yeasts. *Journal of Applied Microbiology, 104*(2), 478–488. https://doi.org/10.1111/j.1365-2672.2007.03566.x

Demyttenaere, J., & Willemen, H. (1998). Biotransformation of linalool to furanoid and pyranoid linalool oxides by *Aspergillus niger*. *Phytochemistry, 47*(6), 1029–1036.

Demyttenaere, J. C. R., del Carmen Herrera, M., & De Kimpe, N. (2000). Biotransformation of geraniol, nerol and citral by sporulated surface cultures of *Aspergillus niger* and *Penicillium* sp. *Phytochemistry, 55*(4), 363–373. https://doi.org/10.1016/S0031-9422(00)00330-7

Esmaeili, A., Hashemi, E., Safaiyan, S., & Rustaiyan, A. (2021, 11/11). Biotransformation of myrcene by *Pseudomonas putida* PTCC 1694. 57.

Esmaeili, A., Rohany, S., & Safaiyan, S. (2012). Biotransformation of citral by free and immobilized *S. Cerevisiae*. *Chemistry of Natural Componds, 48*(2), 88–290.

Eyres, G., & Dufour, J. P. (2009). Hop essential oil: Analysis, chemical composition and odor characteristics. In *Beer in Health and Disease Prevention* (pp. 239–254). Academic Press.

Gibbon, G., & Pirt, S. (1971). The degradation of α-pinene by Pseudomonas PX1. *FEBS letters, 18*(1), 103–105.

Jiang, Z., Xu, C., Wang, L., Hong, K., Ma, C., & Lv, C. (2023). Potential enzymes involved in beer monoterpenoids transformation: Structures, functions and challenges. *Critical Reviews in Food Science and Nutrition, 63*(14), 2082–2092. https://doi.org/10.1080/10408398.2021.1970510

Johnsen, L. G., Skou, P. B., Khakimov, B., & Bro, R. (2017). Gas chromatography–mass spectrometry data processing made easy. *Journal of Chromatography A, 1503*, 57–64.

King, A., & Dickinson, R. (2000). Biotransformation of monoterpene alcohols by *Saccharomyces cerevisiae, Torulaspora delbrueckii* and *Kluyveromyces lactis*. *Yeast (Chichester, England), 16*(6), 499–506. https://doi.org/10.1002/(sici)1097-0061(200004)16:6<499::Aid-yea548>3.0.Co;2-e

King, A., & Dickinson, R. (2003). Biotransformation of hop aroma terpenoids by ale and lager yeasts. *FEMS Yeast Research, 3*(1), 53–62.

Kumar, A., Warburton, A., Silcock, P., Bremer, P. J., & Eyres, G. T. (2023). Yeast strain influences the hop-derived sensory properties and volatile composition of beer. *Foods (Basel, Switzerland), 12*(5), 1064.

Madyastha, K. M., & Murthy, N. (1988). Transformations of acetates of citronellol, geraniol, and linalool by Aspergillus niger: Regiospecific hydroxylation of citronellol by a cell-free system. *Applied Microbiology And Biotechnology, 28*(4), 324–329.

Mäki-Arvela, P., Tiainen, L. P., Gil, R., & Salmi, T. (1997). Kinetics of the hydrogenation of citral over supported Ni catalyst. In *Studies in surface science and catalysis, 108* pp. 273–280). Elsevier.

Mirata, M. A., Wüst, M., Mosandl, A., & Schrader, J. (2008). Fungal Biotransformation of (±)-Linalool. *Journal of Agricultural and Food Chemistry, 56*(9), 3287–3296. https://doi.org/10.1021/jf800099h

Paiva, A. C., & Hantao, L. W. (2020). Exploring a public database to evaluate consumer preference and aroma profile of lager beers by comprehensive two-dimensional gas chromatography and partial least squares regression discriminant analysis. *Journal of Chromatography A, 1630*, Article 461529. https://doi.org/10.1016/j.chroma.2020.461529

Praet, T., Van Opstaele, F., Jaskula-Goiris, B., Aerts, G., & De Cooman, L. (2012). Biotransformations of hop-derived aroma compounds by *Saccharomyces cerevisiae* upon fermentation. *Cerevisia, 36*(4), 125–132. https://doi.org/10.1016/j.cervis.2011.12.005

Richter, T., Silcock, P., Algarra, A., Eyres, G., Capozzi, V., Bremer, P., et al. (2018). Evaluation of PTR-ToF-MS as a tool to track the behavior of hop-derived compounds during the fermentation of beer. *Food Research International, 111*, 582–589. https://doi.org/10.1016/j.foodres.2018.05.056

Rojas, V., Gil, J. V., Piñaga, F., & Manzanares, P. (2001). Studies on acetate ester production by non-Saccharomyces wine yeasts. *International Journal Of Food Microbiology, 70*(3), 283–289.

Rottava, I., Toniazzo, G., Cortina, P. F., Martello, E., Grando, C. E., Lerin, L. A., et al. (2010). Screening of microorganisms for bioconversion of (−)β-pinene and R-(+)-limonene to α-terpineol. *LWT - Food Science and Technology, 43*(7), 1128–1131. https://doi.org/10.1016/j.lwt.2010.03.001

Savithiry, N., Gage, D., Fu, W., & Oriel, P. (1998). Degradation of pinene by *Bacillus pallidus* BR425. *Biodegradation, 9*(5), 337–341.

Schwab, W., Fuchs, C., & Huang, F. C. (2013). Transformation of terpenes into fine chemicals. *European Journal Of Lipid Science And Technology, 115*(1), 3–8.

Sharp, D., Vollmer, D. M., Qian, Y., & Shellhammer, T. H. (2017). Examination of glycoside hydrolysis methods for the determination of terpenyl glycoside contents of different hop cultivars. *Journal of the American Society of Brewing Chemists, 75*(2), 101–108. https://doi.org/10.1094/ASBCJ-2017-2071-01

Siebert, K. (1994). Sensory analysis of hop oil-derived compounds in beer; flavor effects of individual compounds. Quality control. In *Proceedings of the Monograph XXII EBC Symposium on Hops. European Brewery Convention*.

Skov, T., & Bro, R. (2008). Solving fundamental problems in chromatographic analysis. *Analytical and Bioanalytical Chemistry, 390*, 281–285.

Slaghenaufi, D., Indorato, C., Troiano, E., Luzzini, G., Felis, G. E., & Ugliano, M. (2020). Fate of grape-derived terpenoids in model systems containing active yeast cells. *Journal Of Agricultural And Food Chemistry*. https://doi.org/10.1021/acs.jafc.9b08162

Soares-Castro, P., Montenegro-Silva, P., Heipieper, H. J., & Santos, P. M. (2017). Functional characterization of a 28-kilobase catabolic island from Pseudomonas sp. strain M1 involved in biotransformation of β-myrcene and related plant-derived volatiles. *Applied And Environmental Microbiology, 83*(9), e03112–e03116.

Soares-Castro, P., Soares, F., & Santos, P. M. (2021). Current advances in the bacterial toolbox for the biotechnological production of monoterpene-based aroma compounds. *Molecules (Basel, Switzerland), 26*(1), 91.

Stevens, R. (1967). The chemistry of hop constituents. *Chemical Reviews, 67*(1), 19–71.

Steyer, D., Erny, C., Claudel, P., Riveill, G., Karst, F., & Legras, J. L. (2013). Genetic analysis of geraniol metabolism during fermentation. *Food Microbiology, 33*(2), 228–234.

Svedlund, N., Evering, S., Gibson, B., & Krogerus, K. (2022). Fruits of their labour: Biotransformation reactions of yeasts during brewery fermentation. *Applied Microbiology And Biotechnology, 106*(13–16), 4929–4944.

Takoi, K., Itoga, Y., Koie, K., Kosugi, T., Shimase, M., Katayama, Y., et al. (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. https://doi.org/10.1002/j.2050-0416.2010.tb00428.x

Verzele, M., Steenbeke, G., Verhagen, L., & Strating, J. (1989). Preparative liquid chromatography of hop and beer bitter acids. *Journal of Chromatography A, 484*, 361–368.

Warburton, A., Silcock, P., & Eyres, G. T. (2022). Impact of sourdough culture on the volatile compounds in wholemeal sourdough bread. *Food Research International, 161*, 111885. https://doi.org/10.1016/j.foodres.2022.111885

Wright, S. J., Caunt, P., Carter, D., & Baker, P. B. (1986). Microbial oxidation of alpha-pinene by *Serratia marcescens*. *Applied Microbiology And Biotechnology, 23*(3), 224–227.

Yoo, S., & Day, D. (2002). Bacterial metabolism of α-and β-pinene and related monoterpenes by *Pseudomonas sp.* strain PIN. *Process Biochemistry, 37*(7), 739–745.