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The Sensorial and Chemical Changes in Beer Brewed with Yeast Genetically Modified to Release Polyfunctional Thiols from Malt and Hops

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Abstract: The biotransformation of hop aroma, particularly by the cysteine S-conjugate beta-lyase enzyme (CSL), has been a recent topic of tremendous interest among brewing scientists and within the brewing community. During a process often referred to as biotransformation, yeast-encoded enzymes convert flavorless precursor molecules found in barley and hops into volatile thiols that impart a variety of desirable flavors and aromas in beer. Two volatile thiols of particular interest are 3-mercaptohexan-1-ol (3MH) and its acetate ester, 3-mercaptohexyl acetate (3MHA), which impart guava and passionfruit flavors, respectively. In this study, a parental *Saccharomyces cerevisiae* brewing strain that displayed low thiol biotransformation activity was genetically manipulated (GM) to substantially increase its thiol biotransformation potential. Construction of this GM strain involved integration of a gene encoding a highly active CSL enzyme that converts thiol precursors into the volatile thiol, 3MH. Three additional strains were subsequently developed, each of which paired CSL expression with expression of an alcohol acyltransferase (AAT) gene. It was hypothesized that expression of an AAT in conjunction with CSL would increase production of 3MHA. Fermentation performance, sensory characteristics, and 3MH/3MHA production were evaluated for these four GM strains and their non-GM parent in 1.5hL fermentations using 100% barley malt wort hopped at low levels with Cascade hops. No significant deviations in fermentation performance (time to attenuation, final gravity, alcohol content, wort fermentability) or finished beer chemistry were observed between the GM strains and the parent strain with the exception of the speed of vicinal diketones reduction post-fermentation, which was quicker for the GM strains. The GM strains produced beer that had up to 73-fold and 8-fold higher 3MH and 3MHA concentrations than the parent strain, achieving concentrations that were up to 79-fold greater than their sensory detection thresholds. The beers were described as intensely tropical and fruity, and were associated with guava, passionfruit, mango, pineapple and sweaty aromas. These experiments demonstrate the potential of genetic modification to dramatically enhance yeast biotransformation ability without creating off flavors or affecting fermentation performance.

Keywords: thiols; 3-mercaptohexan-1-ol; 3MH; 3-mercaptohexyl acetate; 3MHA; hops; beer; brewing; genetic-modification

Citation: Molitor, R.W.; Roop, J.I.; Denby, C.M.; Depew, C.J.; Liu, D.S.; Stadulis, S.E.; Shellhammer, T.H. The Sensorial and Chemical Changes in Beer Brewed with Yeast Genetically Modified to Release Polyfunctional Thiols from Malt and Hops. *Fermentation* **2022**, *8*, 370. https://doi.org/10.3390/fermentation8080370

Academic Editor: Maurizio Ciani

Received: 8 July 2022
Accepted: 1 August 2022
Published: 5 August 2022

1. Introduction

Over the past two decades, craft beer production in the United States has risen tremendously, with growing demand from consumers for hop-forward beer styles that express strong tropical and fruity flavors. Hop-forward beers are typically achieved by the addition of large amounts of aromatic hops (*Humulus lupulus*) with American pedigree that are added at the end of wort production or during fermentation or post-fermentation [1]. American hop varieties are characteristically fruity and contribute citrus, pine, tropical,

black currant, sweaty and onion/garlic aromas to beer because of the aromatic compounds that originate in the hops' essential oil fraction [2,3]. Of the many compounds found within hop essential oil, which is comprised predominantly of mono- and sesquiterpenes, and to lesser degrees of terpene alcohols, and sparingly of sulfur-containing thiols, the latter are desirable due to their potent contribution to tropical aromas [2,4,5]. While present at extremely low levels relative to other hop-derived compounds [6], volatile thiols have low aroma thresholds in beer, in the range of ng/L [5,7,8], thereby making them potent contributors to a hoppy aroma for some varieties. Three volatile thiol molecules responsible for tropical aromas in beer that have received considerable attention by the winemaking and brewing communities are 3-mercaptohexan-1-ol (3MH), 3-mercaptohexyl acetate (3MHA), and 4-methyl-4-mercaptopentan-2-one (4MMP), which impart distinct aromas of guava, passionfruit, and black currant, respectively [9]. The two thiols of focus in this research, 3MH and 3MHA, have reported sensory detection thresholds in beer of 55 ng/L and 5 ng/L, respectively [7,8].

The free thiol content in hops is strongly varietal dependent [5,10,11] and can be influenced by harvest maturity [12], growing location or terroir [13], and other seasonal variables [8]. American aroma hop cultivars such as Mosaic® and Citra®, which are highly valued for the tropical flavors they impart to beer, have high levels of the free thiols 3MH, 3MHA and 4MMP [14–16]. In contrast to these varieties, many less aromatic hop cultivars produce low or negligible quantities of these thiols in a volatile free form, although their non-volatile precursors may or may not be present [8,9,17]. The basis for these differences in volatile thiol abundance among different hop varietals is not entirely understood, but it likely results from differences in the activities of hop enzymes involved in the thiol biosynthesis process.

The enzymatic steps required for the biosynthesis of volatile thiols in plants have been well studied [18–20]. Most of this research has focused on the biosynthesis of 3MH and 3MHA, as these molecules impart desirable flavors in fermented beverages [9,21]. Biosynthesis of 3MH and 3MHA begins with the degradation of fatty acids by lipoxygenase and hydroperoxide-lyase enzymes, which leads to the production of 3(Z)-hexenal. Next, 3(Z)-hexenal is isomerized to form 2(E)-hexenal by isomerase enzymes [19,21]. Glutathione-S-transferase enzymes conjugate 2(E)-hexenal to glutathione to produce Glut-3MH, which can then be converted to Cys-3MH via transpeptidase enzyme activity [18,22]. Finally, an enzyme possessing beta-lyase activity cleaves Cys-3MH to release 3MH [23]. Subsequent acetylation of 3MH by an alcohol-acyltransferase (AAT) enzyme results in the formation of 3MHA [24].

Differences in the activities of hop enzymes that catalyze each of these steps would result in varying levels of 3MH and 3MHA in hop cones, as well as differences in the abundance of precursor molecules like Glut-3MH and Cys-3MH. Interestingly, several research efforts have revealed that most hop cultivars, even those that produce low levels of 3MH and 3MHA, contain large quantities of thiol precursor molecules like Glut-3MH and Cys-3MH [25–27]. In a survey of eight different hop varietals, Roland et al. [26] found that six of eight hop varietals tested contained no detectable amount of 3MH, but that concentrations of Cys-3MH ranged from 129 µg/kg to 1140 µg/kg, and that Glut-3MH concentrations were even higher, ranging from 1283 µg/kg to 9141 µg/kg. Even amongst hops varietals where 3MH was detected, concentrations of the precursors Cys-3MH and Glut-3MH were >35-fold, and >1500-fold greater than that of 3MH, respectively. These data and those collected by other researchers [27] have made clear that the conversion of these precursors into 3MH is a major bottleneck limiting volatile thiol production in hops.

Interestingly, thiol precursors like Cys-3MH and Glut-3MH have also been found in malt [28], which is the most abundant ingredient in beer production after water [29]. A recent small-scale survey found that barley malts contain up to 700 µg/kg Glut-3MH, while other malts made from rice, wheat, and sorghum contained considerably less, under 30 µg/kg [28]. Among the barley malts, the abundance of Glut-3MH decreased with the extent of malt roasting; no thiol precursors were identified in the most highly roasted malt

analyzed in the study. Further research in this area is needed, as the extent to which these malt-derived thiol precursors impact beer flavor and aroma is currently unclear.

Thiol precursors found in hops and malts have the potential to be converted into volatile thiols during beer fermentation. This conversion requires the activity of yeast-encoded enzymes that perform the terminal steps in the volatile thiol biosynthetic pathway. This process of converting malt and hop-derived thiol precursors into flavor-active thiols like 3MH and 3MHA during beer fermentation is often referred to as "biotransformation" within the brewing community. Due to the enormous potential for biotransformation to enhance desirable tropical fruit flavors in beer and other fermented beverages, this topic has recently received considerable attention.

Most existing yeast strains have a limited ability to release polyfunctional thiols due a 38 bp deletion in *IRC7* gene that encodes for the beta-lyase enzyme [23]. Of those that are capable of biotransformation, only a small proportion of the thiol precursors present in wort are ultimately converted into flavor-active volatile thiols [30]. Experiments with brewing yeast suggest that only 8% of the Glut-3MH and Cys-3MH precursors present during fermentation are converted to 3MH in finished beer [31]. This low rate of conversion has been partially attributed to the yeast beta-lyase enzyme activity, which only weakly catalyzes the conversion of Glut-3MH and Cys-3MH to 3MH [19,31]. The inability of brewing yeasts to catalyze efficient biotransformation results in a significant loss in the potential to produce tropical fruit flavor, as the majority of thiol precursors present during fermentation remain in the flavor-inactive precursor form in finished beer.

The overarching goal of this study was to utilize genetic modification (GM) to create a commercial brewer's yeast such that it would have enhanced biotransformation abilities and be able to release large quantities of tropical thiol molecules from their odorless precursors in malt and hops. We were particularly interested in increasing production of 3MH and 3MHA, as these molecules impart the most highly desirable guava and passionfruit flavors, respectively. To achieve this goal, we first sought to increase 3MH formation by integrating a gene encoding the highly active beta-lyase enzyme, CSL, into the genome of a common brewing yeast strain. Subsequent GM efforts were made to increase the conversion of 3MH to 3MHA. For this, three additional GM strains were created that paired CSL expression with expression of a heterologous AAT enzyme. We hypothesized that the GM strains would display significantly increased 3MH and 3MHA biotransformation ability and produce beer that was characterized as possessing tropical aromas without the presence of offensive/off flavors. We further hypothesized that the fermentation performance of the GM strains would not be significantly altered.

2. Materials and Methodology

2.1. Yeast

The parental yeast strain used as a background for all engineering work was a London Ale strain, BY-London. The genotypes of the engineered strains created in this work are described in Table 1. The integration of expression cassettes encoding CSL and AAT genes into BY-London was performed as follows. DNA encoding CSL and AAT genes MpAAT1 and CATec3 were synthesized by Twist Biosciences. These linear DNAs were cloned into plasmids using the Golden Gate method [32] such that each gene was flanked by yeast-derived promoter and terminator sequences (Table 1). Plasmids also encoded sequences homologous to the yeast FIG2 locus to enable targeted integration of the promoter-gene-terminator expression cassette via homologous recombination. Plasmid DNA was linearized by restriction enzymes, then co-transformed into yeast using a standard lithium acetate transformation protocol alongside a plasmid encoded-endonuclease targeting the FIG2 locus [33]. The endonuclease encoding plasmid also expressed a geneticin resistance gene, conferring resistance to G418. Selection of transformants was accomplished by incubation for 3 days on yeast peptone dextrose (YPD) media containing 200 μg/mL G418. Diagnostic PCR was used to identify yeast transformants in which the expression cassette had been correctly integrated into the FIG2 locus of each homologous yeast chromosome.

Yeast strains were cured of plasmid DNA prior to brewing by serial propagation on YPD media lacking G418.

Table 1. Genotypes of strains created/used in Phase I.

Strain	Background	Genotype
BY-London	-	wild-type
BY-989	BY-London	*FIG2*::*pPGK1*[4]-*CSL1*[1]-*tENO1*[5]
BY-1200	BY-London	*FIG2*::*pPGK1*[4]-*CSL1*[1]-*tENO1*[5], *pHSP26*[4]-*MpAAT1*[2]-*tSSA1*[5]
BY-1201	BY-London	*FIG2*::*pPGK1*[4]-*CSL1*[1]-*tENO1*[5], *pTDH3*[4]-*MpAAT1*[2]-*tSSA1*[5]
BY-1203	BY-London	*FIG2*::*pPGK1*[4]-*CSL1*[1]-*tENO1*[5], *pHSP26*[4]-*CATec3*[3]-*tSSA1*[5]

[1] *CSL* derived from *Gammaproteobacteria*. [2] *AAT* derived from *Malus pumila*. [3] *AAT* derived from *Escherichia coli*. [4] *pPGK1*, *pHSP26*, and *pTDH3* promoters derived from *Saccharomyces cerevisiae*. [5] *ENO1* and *SSA1* terminators derived from *Saccharomyces cerevisiae*.

2.2. Yeast Propagation

Yeast on agar media petri-dishes were shipped overnight from Berkeley Yeast (Oakland, CA, USA) to Oregon State University (Corvallis, OR, USA). Yeast was stored at 1–2 °C until they were inoculated by aseptically transferring a loopful of yeast from each plate to 5 mL of sterilized 10 °P (%*w*/*w*) wort (Briess Golden Light Dry Malt Extract, Chilton, WI, USA). Once transferred, samples were grown at ~22 °C with shaking at ~225 rpm for 24 h. Subsequent propagations occurred under the same conditions at the following increase(s) in volumes; 5 mL to 50 mL, 50 mL to 1 L, and 1 L to 2 L. The 2 L of culture was added to 40 L of aerated 10 °P wort in stainless steel kegs equipped with a pressure relief system along with the addition of 0.1 g/L of yeast nutrient (Yeastex® 82, BSG, Shakopee, MN, USA). The final propagation step was allowed to ferment for three days prior to pitching. A cell count was performed for each strain, and yeast was pitched at a rate of 750,000 cells/mL wort/°P. It is important to note that pitching yeast from the 40 L fermentations into wort resulted in a dilution of approximately 30–40 L (20–27%). This propagation scheme was used to enhance uniformity in growing conditions while also managing eight simultaneous yeast propagations on a pilot scale.

2.3. Brewing

All beers were produced at the Oregon State University pilot research brewery. Wort production was carried out using a split-brew approach, where 300 L of wort was brewed and then split between two fermentations vessels, resulting in ~150 L of wort per treatment. The wort was brewed identically for all treatments, except where hopping timing/rates varied. All wort production was carried out using 100% Rahr Premium Pilsner malt (Rahr Malting Co., Shakopee, MN, USA) with a target starting gravity of 14.8 °P. Wort was boiled for 60 min followed by transferring to a whirlpool separator for hops separation and subsequent cooling via a plate heat exchanger and air infusion prior to transfer to temperature-controlled, stainless-steel fermenters. Post yeast pitching, which was based on the viable cell concentration, starting gravities for the fermentation ranged from 11.5 to 12.0 °P for all trials.

2.4. Hops

All beers were brewed using Cascade hop pellets (Yakima Chief Hops, Yakima, WA, USA). Cascade hops were selected due to their reported high thiol precursor and low free thiol levels [25] to allow for better observation of free thiols contributed by each yeast strains' biochemical pathways, rather than from the free thiols contributed from the hops. The sample of Cascade hops selected for this study had total free and bound thiol contents of 0.009 and 29 μg/g, respectively (quantified by Nyseos, Montpellier, France). Free polyfunctional thiols included 4MMP, 3M4MPol, 3MH, and 3MHA, along with their precursors, cysteine-3MH (C3MH), cysteinylglycine-3MH (CG3MH), γ-glutamylcysteine-3MH (GC3MH), gluthatione-3MH (G3MH), cysteine-4MMP (C4MMP), and gluthatione-

4MMP (G4MMP). A light hopping treatment consisting of 1.48 g/L kettle addition of Cascade hops at the beginning of a 60-min boil was used to compare the thiol production performance of all four GM yeast strains and the parent strain, further denoted as 989, 1200, 1201, 1203, and BYL (parent strain). To observe how an increase in hopping impacted the beer's aromatic profile with a GM strain, a light hopping and whirlpool addition (1.48 g/L kettle addition and 3.00 g/L whirlpool addition just prior to wort chilling) was employed for the GM strain 1201 and parent strain, further denoted as: 1201 + WP and BYL + WP. Strain 1201 + WP was selected in lieu of other GM strains as it showed high 3MH and 3MHA production capabilities in preliminary research. Finally, a heavily hopped treatment was selected for the parent strain consisting of a light hopping, whirlpool and dry-hopping treatment (1.48 g/L kettle addition, 3.00 g/L whirlpool addition, and 10.0 g/L dry hop addition near the end of fermentation), further denoted as BYL + DH. The implementation of increased hopping treatments was performed to compare the sensory characteristics of a beer heavily hopped (i.e., BYL + DH), which resembled a commercial India Pale Ale style, with GM strains that have been lightly hopped. This treatment was employed to compare the sensory characteristics of a beer made with a pronounced dry-hop character to beers made with light hopping but GM yeast designed to release high levels of thiols.

2.5. Fermentation and Finished Beer

All fermentations were carried out at 20 °C in conical, 3 hL (hectoliter) stainless steel fermentation vessels. Fermentations were monitored for pH and gravity (°P) every 12 h until no change in gravity (°P) was observed, after which they were then monitored every 24 h. After the fermentations had attenuated fully, the presence of detectable levels of vicinal diketones (VDK's) was measured by heating a 100 mL sample of the beer to 60 °C in a sealed, glass container, chilling it to room temperature and then assessing the intensity of VDK aroma using orthonasal evaluation by members of the brewing staff. Once this assay yielded no detectable VDK aroma, the fermenter was chilled to 0 °C for 72 h prior to transfer (racking) into 20 L stainless steel sanitized and CO_2 flushed kegs. Beer was directly transferred from the fermentation vessel to kegs via a racking port above the sedimented yeast without the use of filtration. Kegs were stored at 1–2 °C and carbonated (2.6 volumes, 5.2 g/L CO_2) via CO_2 over pressure [34] prior to sensory and chemical analysis. In total, 8 fermentations were carried out with each strain being assigned randomly to their respective fermentation vessel. Fermentations were not replicated in this trial; however, the authors have experience in separate experimentation working with the same hops using the same brewing protocol which were replicated, and these brewing replicates resulted variation in 3MH and 3HA levels with coefficients of variation of only 2.3% and 6.7%, respectively.

2.6. Chemistry

Basic beer chemistry in the form of final gravity (°P), alcohol by volume (%*v*/*v*) (ABV), real extract (%*w*/*w*) (RE), apparent degree of fermentation (%*w*/*w*) (ADF), and real degree of fermentation (%) (RDF) was obtained from an Anton Paar DMA-4500 M-EC with Alcolyzer ME (Anton Paar GmbH, Graz, Austria). All samples analyzed via the Anton Paar instrument were degassed and filtered using Whatman™ 113V filter paper (GE Healthcare, Chicago, IL, USA). pH was measured using a Mettler Toledo SevenEasy S20 pH meter (Mettler Toledo Headquarters, Columbus, OH, USA) and bitterness units were analyzed spectrophotometrically via the American Society of Brewing Chemists Method of Analysis (ASBC MOA) beer-23 (A) [35]. Color measurements were performed using Shimadzu PharmaSpec UV-1700 spectrophotometer (Shimadzu Corporation, Columbia, MD, USA) according to ASBC MOA beer-10 (A) [36]. The Alcolyzer and color measurement assays have coefficients of variation (CV) or relative standard deviation (RSD) of less than 1.5% on replicate measurements, while the BU assay yields data with CV or RSD of less than 4%. Fermentation metrics were characterized as follows: *Time to Attenuation*—time from

start of fermentation to no change in apparent gravity and *Time to VDK pass*—time from attenuation to when sensory detectable levels of VDK's were no longer present.

Quantification of 3MH and 3MHA in beer samples was performed following the derivatizing method developed by Capone et al. [37], with some modifications. 20 mL of each clarified beer sample was combined with 4 mL of 10X phosphate buffered saline, pH 7.4, 200 µL of 10 mM 4,4′-dithiodipyridine (Sigma Aldrich, St. Louis, MO, USA), and 200 µL of 100 mg/mL ethylenediaminetetraacetic acid disodium salt (Research Products International) solution adjusted to neutral pH. The sample was then vortexed for 3 s and incubated at room temperature for 1 h. Thereafter, each sample was extracted with 4 mL of ethyl acetate with vortexting for 20 s. Phase separation was assisted by a brief centrifugation step and 1 mL of the resulting organic phase extract was transferred into a glass vial and dried to completion under nitrogen gas. The resulting thin film of off-white solids was dissolved in 200 µL of LC-MS grade methanol (Thermo Scientific, Waltham, MA, USA), transferred into a 1.5 mL microcentrifuge tube, then clarified by centrifugation at 20,000 rcf for 10 min. The supernatant was transferred into a glass sample vial for analysis by LC-MS.

Chromatography was performed at 35 °C using a reversed-phase C_{18} column (2.1 × 5.0 mm, 1.8 µm pore size, Agilent PN: 959757-902) installed in an Agilent 1290 HPLC equipped with a diode array detector. Derivatized 3MH and 3MHA were detected by mass spectrometry with selected ion monitoring at 244.4 M/Z and 286.4 M/Z, respectively, using an in-line Agilent 6130 single-quadrupole mass spectrometer. Derivatized 3MH eluted at 5.3 min under a linear 5–40% over 12 min acetonitrile gradient supplemented with 0.1% (*v*/*v*) formic acid; derivatized 3MHA eluted at 8.5 min. Quantification was accomplished by comparing peak areas to standard curves generated by spiking known concentrations of 3MH and 3MHA into beer brewed with BY-London, then following the same derivatization and analyte extraction protocol as detailed above.

2.7. Sensory Evaluation

Two methods of descriptive sensory analysis were performed: a Check-All-That-Apply (CATA) approach [38] and line scaling [39]. All subjects gave their informed consent for inclusion before they participated in the sensory analysis, and approval for this work was granted by Institutional Review Board at OSU (Study Number IRB-2019-0247). Both sensory methods were performed during a single sensory session, where all beer samples were assigned random three-digit blind codes and presented to evaluators in a panelist-specific random order. Samples were presented in ~60 mL aliquots in lidded, black plastic cups to eliminate potential visual differences. Panelists were instructed to first smell each sample in the order presented and perform the CATA evaluation. Post CATA evaluation, panelists were instructed to perform line scaling on the same sample before moving on to the next sample. A two-minute forced wait was implemented to aid in reducing panelist fatigue. Two separate data collection instruments consisting of a palette of sensory attributes for the CATA evaluation (Table S1) and a line scale (no aroma—extreme aroma) were built using Qualtrics software (Qualtrics, Provo, UT, USA) and administered using Chromebook tablet computers. The sensory panel consisted of 18 panelists (10 female and 8 males; age range 24–67 years old) with prior experience performing descriptive analysis on wine and beer and who were pre-screened prior to testing for any smell or taste defects and demographic information. Panelists were trained during a separate training session prior to data collection as described below.

The CATA approach used a list of 19 attributes (fruity, resinous, melon, tropical, sweaty, stone fruit, guava, passionfruit, mango, pineapple, herbal, vegetal, grainy, citrus, floral, nutty, caramel, grassy, and earthy) which were generated by a preliminary evaluation of all treatments using a subset of the sensory panel. The list of attributes was created to encompass both generic and specific terms that may or may not describe each sample. Panelists were instructed to smell each sample orthonasally and select all the attributes that they felt best described each sample. Panelists were trained on each attribute during a

separate training session, during which food and chemical reference standards (Table S1) were examined for all the listed attributes.

Attribute scaling was performed for two attributes: overall tropical aroma intensity and overall hop aroma intensity (OHAI). Each attribute scale consisted of line scale with anchors *no tropical aroma* or *no hop aroma* on the left end and *extreme tropical aroma* or *extreme hop aroma* on the right, for each respective scale. Panelists used a slider bar to indicate the attribute intensity on the line scale, and they were trained on each scales' anchors by using an American Light lager (Bud Light AB-InBev, St. Louis, MO, USA) to represent no tropical aroma and a tropical fruit juice (Pineapple Orange Guava juice, Meadow Gold Dairies, Honolulu, HI, USA), as extreme tropical aroma. Similarly, the American Light lager served as the no OHAI anchor and a double IPA from a local brewery (Sticky Hands India Pale Ale, Block15, Corvallis, OR, USA) was used as extreme OHAI. During the practice session, panelists were given these external standard samples to evaluate, and they collectively came to agreement on where each sample fell on the two different line scales.

2.8. Statistical Analysis

All statistical analyses, analysis of variance (ANOVA), Fisher multiple comparisons, Cochran's Q test, and correspondence analysis (CA) were performed using XLSTAT with the corresponding Sensory Analysis Package (Addinsoft, v. 2020.5.1). All CATA frequency data was first analyzed using Cochran's Q test to determine which attributes were significant at differentiating among samples. Significant attributes and attributes that were used >30% of the total maximum responses were re-analyzed via correspondence analysis, the results of which are presented in the form of biplots. This same approach was applied to only the genetically modified (GM) strains by removing the subset of parent strain and increased hopping rates to observe the differences among the GM strains alone.

Attribute intensity scaling data was analyzed via two-factor analysis of variance (ANOVA) with the response representing the tabulated line scaling results for each attribute with panelist treated as a random factor and yeast/hop treatments as fixed factors. Post-hoc analysis via Fishers Least Significant Difference test (Fishers LSD) was performed on all ANOVA results that identified significant differences between samples.

3. Results and Discussion

3.1. Beer Chemistry and Fermentation Performance

An important consideration with new strain selection for alcoholic fermentations, whether it be via modifying existing strains, as in this study, creating new hybrids or collecting environmental strains, is their fermentation performance as characterized by chemical, physical and sensorial outcomes. While the key focus of this study was to evaluate GM strains displaying improved ability to release 3MH from their cysteine-conjugated precursors and to acetylate 3MH to 3MHA, observing and characterizing their fermentative performance compared to their parent strain was essential in establishing that their use would be suitable in a commercial setting, regardless of the extent to which they generate free thiols. Furthermore, fermentation performance is an important performance metric in terms of fermentation/beer throughput in a brewery's cellar and the flavor qualities of the finished beer.

All strains in this study fermented a 100% barley malt wort with negligible differences in final gravity, alcohol content, residual/real extract (RE), wort fermentability (apparent and real degree of fermentation, ADF & RDF), and time to attenuation (Table 2 & Figure S1). The final apparent gravity ranged from 4.1 to 4.4 °P for all fermentations, resulting in real degree of fermentation (RDF) values ranging from 54 to 57%. While the final gravities were higher and the RDF's lower than anticipated, they still fell within the range of acceptability [40]. While the GM strains performed similarly to the parent strain for all of these metrics, two notable differences were observed. One was associated with time to attenuation for the late-hopped treatments and the other was the time to reduce vicinal diketones post-fermentation. With regard to late-hopping treatments with the parent

strain and GM strain 1201, wort production for three of the trials differed from the other five in that the wort received a second, late-hop addition during whirlpool separation, BYL + WP, 1201 + WP, and BYL + DH, and the last treatment (BYL + DH) also received a third hop dose at the end of fermentation. These late/dry-hop treatments were performed to provide higher hop aroma comparisons to the low/kettle-only hopping treatments. In these three cases where whirlpool hopping was added during wort production, the time to attenuation during fermentation averaged 143 h compared to 93 h for the 5 non-whirlpool hopped treatments.

Table 2. Finished beer chemistry and fermentation performance.

Analysis	Strain							
	989	**1200**	**1203**	**1201**	**1201 + WP**	**BYL**	**BYL + WP**	**BYL + DH**
Final Gravity (°P)	4.4	4.4	4.2	4.3	4.3	4.2	4.1	4.4
ABV (%*v*/*v*)	4.7	4.7	4.9	4.9	4.8	4.9	5.0	5.1
Real Extract (%*w*/*w*)	6.3	6.4	5.9	6.3	5.9	6.2	6.2	6.3
ADF (%*w*/*w*)	66	65	69	67	68	67	68	68
RDF (%)	55	54	57	55	57	56	56	56
BU	20	21	18	27	31	17	32	44
Final pH	4.3	4.6	4.5	4.5	4.6	4.6	4.7	4.8
Color (ASBC)	5.6	6.4	7.5	7.7	5.7	5.6	6.1	7.1
Time to attenuation (h)	75	99	96	120	137	77	137	151
Time to VDK pass (h)	85	120	133	134	120	205	168	96

Another important fermentation performance characteristic is the length of time yeast take to reduce the concentrations of vicinal diketones (VDK, diacetyl and 2,3 pentanedione) to levels below human sensory detection levels (approximately 20–30 µg/L) [41] after the fermentation attenuates. This was a parameter where differences were noted between the parent strain and the GM strains. The BYL parent strain was the slowest to reduce VDKs, taking 205 h, while GM strain 989 was the fastest VDK reducer (85 h). The other three GM strains reduced VDK in 120–134 h. This difference could prove significant for a commercial brewery concerned with fermentation and post-fermentation speed, and it highlights further potential benefits of using some of the GM strains, however, further studies need to be performed to draw a definitive conclusion on enhanced VDK reduction performance by the GM versus parent strain.

While not a fermentation performance characteristic, the late/dry-hopping treatments also resulted in an increase in the bitterness units (BU), which was anticipated. Within the GM strains hopped at the same rate, 1201 exhibited the highest BU value (27) while 1203 had the lowest (18). While the BU technique is a modestly imprecise analytical technique, the large difference between these two strains is mostly unexplained. We speculate that it could be due to factors such as differences in yeast cell surface hydrophobicity and or foam production/stability, both of which can impact the degree of hop acids scalping from fermentation media.

3.2. Thiol Production

All four of the GM strains in this study produced substantially more 3MH and 3MHA compared to the parent strain regardless of hopping regime (Figure 1). The 3MH concentrations produced by the GM strains ranged from 2677 to 4354 ng/L, where values for the parent strain (within all hopping treatments) were below our 50 ng/L quantification threshold, apart from BYL + DH (3MH concentration of 144 ng/L). Within the GM strains, 989 produced the highest amount of 3MH and 3MHA (4354 and 162 ng/L, respectively) while 1200 produced the lowest (2677 and 56 ng/L, respectively). With sensory detection thresholds for 3MH at approximately 55 ng/L and 3MHA at 5ng/L in beer [7,8], the GM strains produced free thiol levels that were up to 79-fold greater than reported sensory detection thresholds and >87-fold greater that of the parent strain BYL.

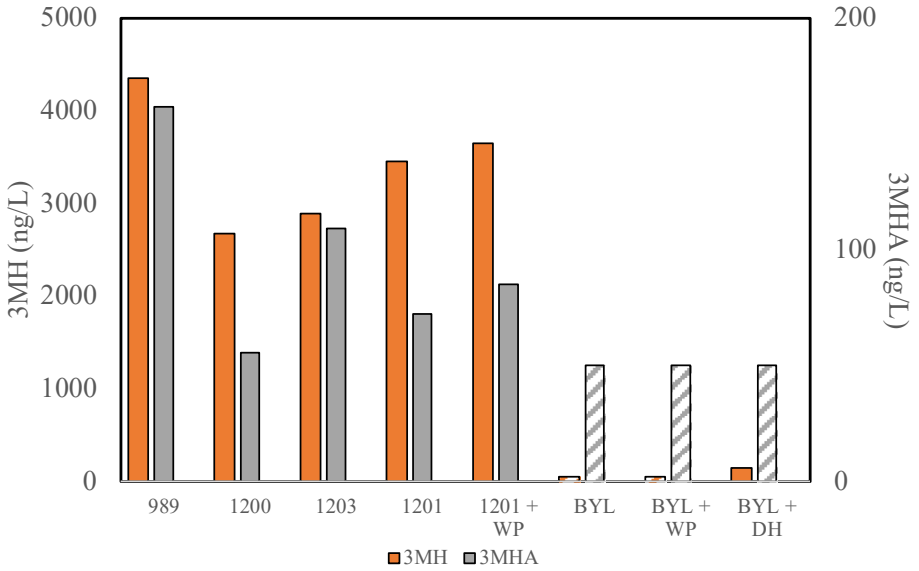

Figure 1. Thiol concentrations (ng/L) of 3MH and 3MHA in beer as a function yeast strain and hop treatment. Dashed columns represent the presence of either 3MH (orange) or 3MHA (gray) at concentrations below the quantification limit of 50 ng/L. Values represent averages of duplicate analyses where the coefficient of variation for all measurements averaged 4.2% (ranging from 0.2 to 11.9%).

These data indicate that the malt used for these fermentations, in addition to the relatively light kettle hop addition, contained a substantial quantity of thiol precursors which were converted into 3MH and 3MHA by the GM strains. Whirlpool hopping of the 1201 fermentation also increased the concentrations of 3MHA at least 2-fold compared to a non-whirlpool hopped treatment, providing evidence that thiol precursors may also be supplied by whirlpool hop addition. Interestingly, 3MH concentrations were largely unaffected by whirlpool hop addition. Although future research will be required to tease apart the relative contributions of malt and hop derived precursors at each stage in the fermentation process, these data make clear that GM strains in this study catalyze a substantial increase in the conversion of these precursors into volatile thiols.

Interestingly, the GM strain engineered to express CSL alone (989) produced more 3MH and 3MHA than any of the GM strains that expressed both CSL and a heterologous AAT (1200, 1201, 1203). This was unexpected, as we had hypothesized that AAT expression would likely increase 3MHA concentration while decreasing 3MH concentration, due to the AAT-catalyzed conversion of 3MH to 3MHA. The cause of this decrease in 3MH and 3MHA concentration associated with heterologous AAT expression is not immediately clear. A potential explanation is that both AATs utilized in this work possess thiol degrading activity that directly reduced the concentrations of 3MH and 3MHA in beer. This seems unlikely however, given that AAT activity has been well studied, and no mechanism has been discovered through which these enzymes would be capable of degrading 3MH [42]. Alternatively, the reduction in 3MH and 3MHA by strains expressing heterologous AATs could have been caused by indirect means. In these strains, the expression cassettes encoding the AATs were integrated into the BY-London genome immediately adjacent to the CSL expression cassette. Although CSL and the AAT expression were driven by distinct promoter sequences, the presence of the AAT-encoding cassette likely altered CSL expression through modulation of chromatin dynamics [43], or by titrating available transcription factors [44]. Such effects could have decreased CSL expression relative to its expression in 989, leading to a decrease in production of 3MH, and a consequent decrease in 3MHA. Additional research is needed to investigate whether this was the case.

Although strains 1200, 1201, and 1203, produced lower concentrations of 3MH and 3MHA than 989, the relative ratios of these three thiols differed amongst all strains. For example, the molar ratio of 3MH to 3MHA in beer brewed strain 1200 was ~63:1, whereas

for beer brewed with 989 it was ~35:1. Given the differences in aroma and perception threshold between these two volatile thiols, these varying ratios likely impacted the sensory perception of these beers, as described in detail below.

3.3. Sensory Characterization

Sensory analysis was performed on all beers to characterize their aromatic profiles and assess the effects of increased 3MH and 3MHA concentrations. Initial screening of the finished beer aromas did not reveal any offending aromas, thus highlighting the potential of the strains to enhance production of specific desirable flavor molecules without concomitant increases in off-flavor molecules. In particular, the lack of ethyl acetate-related aromas, such as solventy or nail polish, indicated that the AATs expressed by these strains did not produce substantial quantities of this off-flavor molecule, as yeast-encoded AATs ATF1 and ATF2 are known to do [45,46]. While ethyl acetate was not quantified instrumentally, its concentrations were below that detectable by the sensory panel.

The results from the sensory descriptive CATA frequency data identified five attributes that were significant in differentiating the samples: tropical, passionfruit, resinous, citrus and herbal (Cochran's Q test, p-value < 0.05). These attributes, as well as those used >30% of the maximum frequency of all responses (guava, sweaty, fruity, pineapple, mango), were collectively analyzed via correspondence analysis (CA). The CA biplot shows all yeast strains and their relation to their corresponding attributes (Figure 2A) as well as a reanalysis of just the GM strains without the inclusion of the parent strain and/or increased hopping rates (Figure 2B). The latter analysis was performed to gain better insight into the specific sensory attributes, passionfruit, and guava, produced by the GM strains.

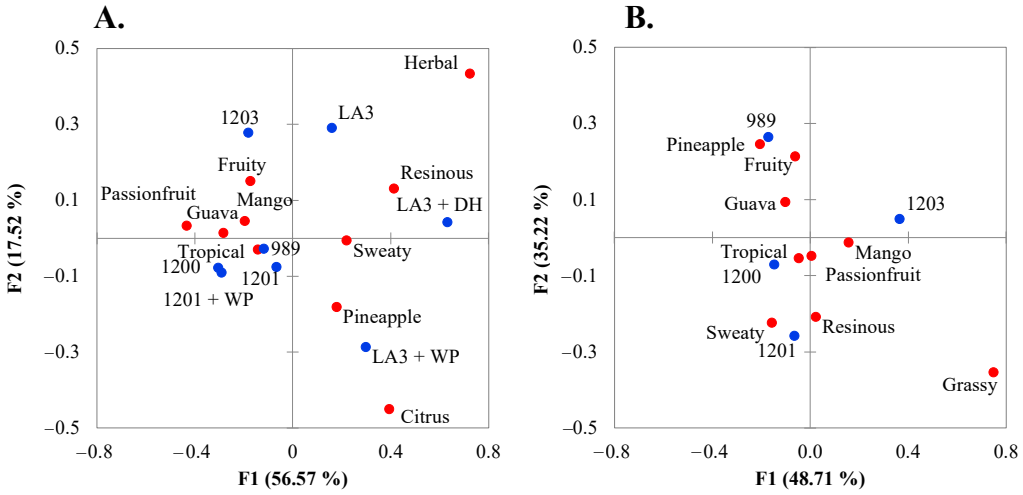

Figure 2. Correspondence analysis of sensory CATA data for all yeast strains (**A**) and genetically modified strains excluding parent strain and varied hopping treatments (**B**).

When examining all yeast and hop treatments in total (Figure 2A), there is a clear distinction between non-GM and GM strains, with all GM strains falling to the left of the Y-axis in a region associated with fruity and various tropical fruit aromas such as passionfruit, guava, mango, and general tropical aroma, and all non-GM strains falling to the right with lower frequencies of the tropical aromas and greater frequencies of herbal, resinous, pineapple and citrus. As anticipated, the dry-hopped parent strain was strongly associated with resinous and sweaty, both common characteristics identified with dry-hopped beers. BYL was more strongly associated with herbal and fruity aromas while the addition of late, whirlpool hopping (BYL + WP) resulted in a greater frequency of pineapple and citrus descriptors. The biplot also reveals similarities between 1201 and 1201 + WP, which suggests that the potency of the free thiols dominated the aromas in the GM strains regardless of whether additional hopping (i.e., whirlpool addition in this case) was performed.

Closer inspection of the GM strain biplot (Figure 2B) along with the raw data in Table S2, identified strain 989 as presenting a high frequency of pineapple and fruity descriptors. This strain produced the greatest amounts of 3MH, a thiol often characterized as guava [47], and 3MHA, which smells of passionfruit [48]. Strain 1200 had the greatest frequency of generic tropical aroma, 1201 with resinous, and 1203 with mango (although not statistically significant). All four GM strains presented high frequencies of generic tropical aroma. The somewhat similar tropical profiles, albeit with nuanced differences, may be due to the high levels of free 3MH and 3MHA. Strain 989, in particular, produced beers containing a very high concentration of 3MH, greater than 71 times the aroma detection threshold. At these high levels, these compounds may have saturated the aromatic profile with thiol-derived aromas. Additionally, there are other fermentation-derived molecules such as volatile esters and higher alcohols that are likely to interact with the thiols to create complex flavor profiles that cannot be fully described by looking solely at the concentration of a singular compound [49]. Regardless, there is overwhelming evidence that all GM strains were able to release polyfunctional thiols present in the Cascade hops and/or malted barley to create beers containing high levels of 3MH and 3MHA, and which had intensely tropical aromas.

While the CATA data provide a useful qualitative map of the various treatments, the technique does not inform the degree of intensity among the treatments for a given attribute. Therefore, attribute scaling was implemented to determine the intensity of tropical and overall hop aromas to quantify their relative intensities among the yeast and hop treatments. While tropical aromas can be classified as hop-derived, for instance in hops that have high free thiol content, such as the variety Mosaic®, in this study, panelists were instructed to evaluate OHAI and tropical aroma intensities separately to distinguish the thiol-driven, tropical aromas separate from the resinous, fruity and herbal qualities found in hops (i.e., terpenes, sesquiterpenes, and terpene alcohols).

Analysis of variance of the attribute scaling data identified panelists and yeast/hop treatments as significant factors for both attributes (Table 3). A significant panelist effect is to be expected in a sensory panel scaling attribute intensity with limited training [50]. In terms of tropical aroma intensity, the panel found all GM strains to be significantly higher than the parent strain regardless of hopping rate/timing (Figure 3A), with the exception of strain 1201, which did not differ significantly from BYL + WP (Fisher's LSD, p-value < 0.05). Whirlpool hop addition to strain 1201 modestly increased tropical aroma intensity compared to 1201 without whirlpool hop addition, but this effect was not statistically significant.

Table 3. Analysis of variance overall hop aroma intensity (OHAI) and tropical aroma intensity.

Source	Degrees of Freedom	OHAI		Tropical Aroma Intensity	
		F	*p*-Value	F	*p*-Value
Panelist	17	6.52	<0.0001 *	2.43	0.003 *
Sample	7	8.56	<0.0001 *	6.23	<0.0001 *

* Statistically significant.

Panelists found the dry-hop treatment to be significantly more aromatically hoppy than all other treatments, as evidenced by the significantly increased OHAI attributed to beer brewing with BYL and dry-hop addition (Figure 3B). There were modest to no significant differences among the GM strains in perceived OHAI, except for 1201 which was significantly higher than 1203. Whirlpool hop addition to strains 1201 and BYL did not result in significant differences in OHAI or overall tropical aroma intensity, indicating that any effects of whirlpool hopping on flavor were not captured by the OHAI metric.

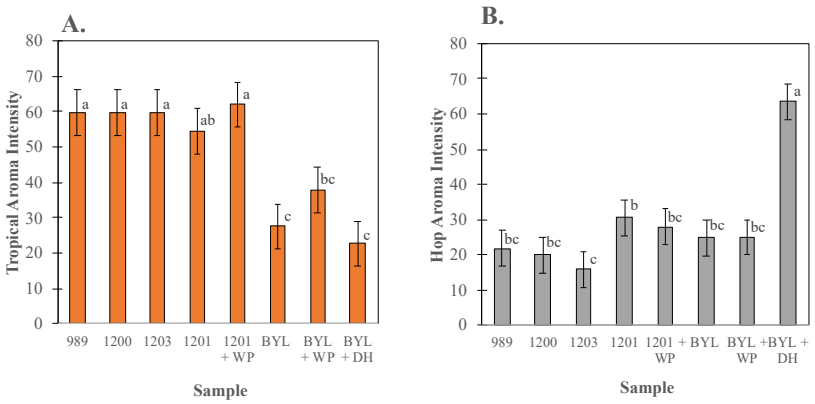

Figure 3. Tropical aroma intensity (**A**) and overall hop aroma intensity (**B**) from attribute scaling data (0–100 scale). Error bars represent standard error and data labels that share a letter (a, b, c) are not significantly different (Fishers LSD, p value < 0.05).

These data suggest that the two metrics used for aroma intensity evaluation, tropical aroma intensity, and OHAI, appear to be unrelated in this study. This is evidenced by the observation that all GM strains display high tropical aroma intensity and low OHAI, whereas the BYL parent strain with dry-hop sample was described as having low tropical aroma intensity and high OHAI. This observation was somewhat expected given our study design, as Cascade hops are known for imparting floral, citrus, and herbal flavors, whereas 3MH and 3MHA impart distinct flavor notes associated with guava and passionfruit flavors. If the BYL fermentation had been dry-hopped with a hop variety that contained significant concentrations of 3MH and 3MHA, e.g., like Citra® or Mosaic®, the flavor profile may have been more similar to that of the beer brewed by the GM strains.

These data also report that beer brewed with each of GM strains was described as having a similar tropical aroma intensity. This was surprising, given that the concentrations of 3MH and 3MHA varied considerably among the different beers. For example, beers brewed with 989 and 1200 had nearly identical tropical aroma intensities, even though 989-derived beer contained 1.6- and 2.9-fold more 3MH and 3MHA, respectively. These data suggest that the thiol concentrations, particularly for 3MH, may be reaching their terminal threshold concentrations. Terminal thresholds are a well-established concept in sensory analysis and refer to the concentration at which stimuli no longer elicit an increased response [51]. Identifying terminal threshold values for these thiols in beer may prove to be beneficial in further GM studies, as a known terminal concentration would allow yeast developers to target thiol concentrations below their terminal threshold levels rather than attempting to promote thiol expression to the greatest possible extent. As reported in Figure 2 above, clear differences in aroma were still observed among the GM strains, especially between 989 and 1203 which were associated with guava/pineapple, and mango aromas, respectively. Further investigation is required to determine whether these flavor differences are due to differences in 3MH/3MHA concentrations or interactions among other flavor molecules, as complex matrices and their role in influencing perceived aroma in beverage products [49,52] could be influencing the perception of specific tropical aroma attributes in these samples.

4. Conclusions

The overall goal of enhancing thiol expression in beer through the genetic modification of a brewing-specific, commercial yeast strain (*S. cerevisiae*) was achieved, with 3MH and 3MHA levels reaching up to 87- and 3.22-fold higher, respectively, than what was produced in the parent strain and up to 79-fold greater than their sensory detection threshold concentrations. The substantial increase in free thiol production in the new strains resulted in a significant increase in overall tropical aroma and specific, thiol-driven aromas such as guava, passionfruit, and mango along with other tropical and fruity aromas in contrast to

the parent strain. The thiol concentrations in beer produced with the GM strains varied 1.62-fold for 3MH, and 2.9-fold for 3MHA.These high concentrations of volatile thiols in all beers brewed with GM strains were likely responsible for these beers being associated with similarly "tropical" flavor notes. Nevertheless, clear flavor and aroma differences among these beers were also observed, most notably between the guava dominated flavor of beer brewed with 989, and the stronger mango flavor associated with beer brewed with 1203. The successful modification of a low thiol producing parent strain to one that has pronounced beta-lyase and AAT activities opens the door to producing beer with strong and varied tropical flavors and aromas. These strains used in conjunction with various malts and hopping techniques could be extremely useful in creating new beer flavors and increasing the number of tools brewers have at their disposal for producing a diverse array of beer flavors. The substantial increase in biotransformation capacity displayed by these strains also opens the door to discovering additional recipe and process modifications that further impact the biotransformation process and resulting beer flavors. Research that identifies hop and barley varieties containing varying concentrations of thiol precursors will clearly be fundamental in understanding how best to utilize these strains. Investigations into how to pair these strains with specific hop varietals to either increase the complexity of beer flavors, or to simply boost the intensity of tropical flavors will also be of great value. In summary, these GM strains represent a valuable new resource for the brewing community that could have direct impacts on beer flavor and that will also facilitate future biotransformation research efforts.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8080370/s1, Figure S1: Fermentation performance data of all yeast strains; Table S1: Check-All-That-Apply Attributes and Training Standards; Table S2: Frequency of the Aroma and Flavor Attributes Selected by the Panelists (N = 18) during CATA Sensory Evaluation of Beer Samples.

Author Contributions: The authors confirm contributions to the article as follows: study conception and design: J.I.R., C.M.D. and T.H.S.; genetic transformation of strains used in this study: C.J.D. and S.E.S.; data collection: R.W.M., C.J.D. and D.S.L.; analysis and interpretation of results: R.W.M., J.I.R., C.J.D. and D.S.L.; draft manuscript preparation: R.W.M., J.I.R., C.M.D., D.S.L. and T.H.S. All authors have read and agreed to the published version of the manuscript.

Funding: This work was made possible by federal research funds from USDA-NIFA-SBIR-007712 award number 2021-33530-34357.

Institutional Review Board Statement: All subjects gave their informed consent for inclusion before they participated in the sensory analysis, and approval for this work was granted by Institutional Review Board at Oregon State University (Study Number IRB-2019-0247).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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