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Engineering Industrial Brewing Yeast for Flavor Production

by

Rachel A. Li

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Plant Biology

in the

Graduate Division

of the

University of California, Berkeley

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Spring 2018

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by Rachel A. Li

Abstract

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Doctor of Philosophy in Plant Biology

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Yeast has been used by various peoples for thousands of years to make beer, wine, and other alcoholic beverages. During fermentation, yeast impart a host of flavor and aroma molecules to wine and beer, derived from both central and secondary metabolism. Many of these compounds are derived from the interactions between yeast enzymes and compounds contributed by hops, in the case of beer, and grapes, in the case of wine. The field of metabolic engineering has opened up new avenues for flavor production in microbes such as yeast. In addition to the overexpression or deletion of yeast endogenous enzymes responsible for flavor modulation, *de novo* production of flavors in yeast is also possible. In the work presented in this doctoral dissertation I describe how these tools have been applied to the engineering of brewer's yeast strains in order to reduce, and perhaps eventually replace, aromatic hop additions used in the brewing process.

Chapter 1 presents a review of genetically engineered brewing and wine yeasts. Commercial and academic examples are discussed. Traits that have been engineered encompass flavor production and off-flavor reduction, flocculation, sugar consumption, and other fermentation performance characteristics.

Chapter 2 presents my work engineering California Ale Yeast to biosynthesize the compounds linalool and geraniol, which impart hoppy flavor and aroma to beer. At least one engineered strain was shown to produce beer perceived as hoppier than traditionally hopped beer by a sensory panel in a double-blind tasting.

Chapter 3 describes additional engineering of the monoterpene-producing strains and suggests future work to more fully reconstitute the complex flavor imparted by traditional hop additions. Engineered strains provide an avenue for improvement and innovation of the brewing process by making it possible to generate new and distinctive flavors with consistency and reproducibility.

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1. Chapter 1 - Customization of wine and brewing yeast strains by genetic engineering

1.1. History

The unicellular fungus known as yeast has been used by various peoples for thousands of years to make beer, wine, and other alcoholic beverages. For most of that history, brewers and winemakers were unaware that yeast (and other microbes) were responsible for fermentation, or indeed even present. Found naturally on ripe, especially sugar-rich fruit, these microorganisms cause spontaneous fermentations in the presence of fermentable sugars and low oxygen environments. It is likely that the first alcoholic fermentations conducted by humans were accidental; for beer, grains and for wine, grapes, might have been left to sit and wild yeasts in the surrounding environment or covering the fruit would have started fermenting. The resulting drink may have been considered spoiled, but evidence for deliberate wine production (although likely also via spontaneous fermentation) dates as far back as 4000 B.C. to ancient Armenia, Egypt, and Greece; brewing dates as far back as ancient Iran, Mesopotamia, and Egypt. Over time, the process became more controlled; new fermentations were inoculated with the remains of a previous fermentation, which - without the knowledge of the brewers and winemakers - transferred the yeast from batch to batch and selected for yeasts with desirable fermentation characteristics.

For hundreds of years, beer and wine were made without a full understanding of the fermentation process, the conversion of sugar into alcohol and other byproducts. Then in the late 17th century, Anton van Leeuwenhoek, an early (and often credited as the first) microbiologist and designer and builder of microscopes, observed yeast cells under one of his microscopes. Despite his discovery of these “globules” in beer, he did not realize that they were living organisms and instead believed them to be derived from the malt¹. In the late 18th century, Antoine-Laurent Lavoisier defined fermentation as the conversion of sugar into alcohol and carbon dioxide. However, he left out the causative agent, yeast. Finally, in the mid 19th century, Louis Pasteur concluded that yeast was responsible for fermentation, that it was a living organism, and that bacteria and other yeast species lead to spoilage of beer and wine. His work led to the first isolation of pure yeast cultures and was the foundation for improved control over the beer and winemaking processes.

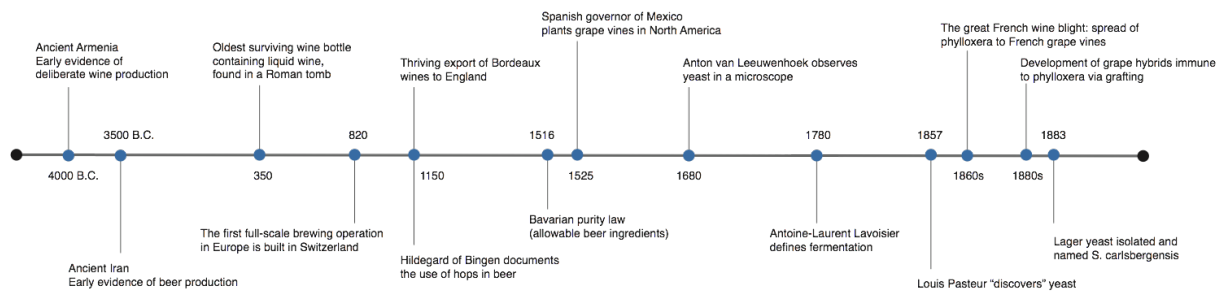


Figure 1.1. History of wine and beer production and related scientific discoveries. The “discovery” of yeast brought both industries into the modern era and gave the winemaker and brewer better control over the fermentation process.

1.2. Strains

The most commonly used yeast species in winemaking is *Saccharomyces cerevisiae*, which converts the sugar found in grapes, mainly glucose and fructose, into ethanol and carbon dioxide, in the process also releasing various flavor-contributing molecules. *S. cerevisiae* is also used in brewing, where it is known as ale yeast, or “top-fermenting” yeast. It converts the fermentable sugars from barley, mainly maltose, but also maltotriose and glucose, into ethanol, carbon dioxide, and various flavor-contributing molecules. A second *Saccharomyces* species is also used in brewing: *S. pastorianus*, known as lager yeast, or “bottom-fermenting” yeast, which was originally isolated at the Carlsberg brewery in Copenhagen, Denmark and given the name *S. carlsbergensis*. Strains of *S. cerevisiae* (and other industrial microbes) have adapted over time to the specific processes in which they are used, as brewers or winemakers reused the yeast from favored batches and selected for preferred traits.

New yeasts have also been generated by hybridization. This occurs both naturally - the lager yeast *S. pastorianus* is itself a hybrid between *S. cerevisiae* and *S. eubayanus* - and in the laboratory. Work by Steensels et al generated strains with increased isoamyl acetate production, a key aroma compound described as fruity/banana-like and particularly desired in fruity ales, by crossing different *S. cerevisiae*, *S. paradoxus*, and *S. pastorianus* yeast strains². Mertens et al hybridized several strains of *S. cerevisiae* and *S. eubayanus* to generate new *S. pastorianus* lager strains³. Several exhibited broader temperature tolerance than their parents and the *S. pastorianus* reference strain and desirable aroma production. Hybridization between various species of yeasts has the potential to generate improved strains; however, cross-breeding is limited to traits associated with native yeast metabolism. In addition, the transfer of whole genomes via hybridization, rather than the incorporation of a single gene using molecular biology techniques, may introduce undesired traits that must be removed by multiple generations of backcrosses.

Recent advances in genetic engineering allow for the introduction of modifications more targeted than by hybridization, and the ability to add as little as a single gene may circumvent the introduction of undesired traits. These techniques have been used to generate a few commercially available industrial strains, as well as a host of academic strains with commercial applications, all derived from *S. cerevisiae*. The

introduction of non-*cerevisiae* DNA into the *S. cerevisiae* genome categorizes the resulting strains as transgenic⁴. Despite the handful of examples, most companies have avoided bringing such strains to market for fear of consumer backlash against genetically modified (GM) organisms (although this has yet to be tested; it is still unclear how consumers will respond to GM yeast). In the United States, however, there is no difference in labeling requirements for GM and non-GM products. Yeast, even GM yeast, is categorized as a processing aid with Generally Regarded As Safe (GRAS) status when used as intended⁴; therefore beverages made with GM yeasts are exempt from being labeled as such and wineries that use GM strains are not required to report their use. In addition, strains containing DNA derived from the same species, or a related species that can exchange genetic material with the host organism by natural processes, are not considered transgenic but rather “self-cloned.”

1.3. Commercial GE strains

Three genetically modified *S. cerevisiae* strains have been approved for sale in the United States and Canada and are commercially available for use in winemaking. One strain is transgenic (it contains heterologous DNA from non-yeast species and is classified as a genetically modified organism, or GMO); the other two are “self-cloned” and are therefore non-transgenic⁴. In the US, all three have received GRAS status from the Food and Drug Administration (FDA). The first strain to receive GRAS status, ML01, was developed by Lesaffre International in collaboration with the University of British Columbia (the laboratory of Dr. Hennie van Vuuren) and the University of Guelph (the laboratory of Dr. Ron E. Subden)⁵. This strain, with genes from *Schizosaccharomyces pombe* (encoding malate permease) and *Oenococcus oeni* (encoding a malolactic enzyme), is capable of malolactic fermentation, a secondary fermentation used to convert tart-tasting malic acid into the milder lactic acid⁶. Malolactic fermentation is commonly used in the production of most red wines and some white wines and is carried out by lactic acid bacteria, including *Oenococcus oeni*, which are added after the primary yeast fermentation, thereby increasing the total fermentation time⁷. The use of the ML01 strain, however, negates the bacterial addition and the secondary fermentation, decreasing the total fermentation time and avoiding off-flavors generated by the lactic acid bacteria during the often unpredictable process. As it contains genes from non-yeast organisms, this strain is considered to be a GM organism.

The second strain, ECMo01, was developed at the University of British Columbia in the laboratory of Dr. Hennie van Vuuren^{5,8}. This strain contains an extra copy of the yeast *DUR1,2* gene (encoding a urea amidolyase), regulated by the high-strength PGK1 promoter. This affects ethyl carbamate production, a byproduct of fermentation formed by the reaction of ethanol and urea and a known carcinogen. Overexpression of urea amidolyase increases urea degradation, leading to a reduction in ethyl carbamate. Since the introduced gene is a native yeast gene, this “self-cloned” strain is considered to be non-transgenic.

The third strain, P1Y0, was developed by Phyterra Yeast Inc. in collaboration with Dr. Linda Bisson at the University of California at Davis⁵. The native *MET10* allele in this strain has been replaced with an allele of the *MET10* gene obtained from the wine strain UCD932. Expression of this allele leads to a reduction in hydrogen sulfide

formation, a byproduct of fermentation and an off-flavor in wines, an effect exhibited in a variety of strain backgrounds⁹. Since the introduced gene in P1Y0 is a native yeast gene, this “self-cloned” strain is also considered to be non-transgenic.

1.4. Academic examples: flavors

During fermentation, yeast impart a host of flavor and aroma molecules to wine and beer, derived from both central and secondary metabolism. Many of these compounds are present at very low concentrations, some below their thresholds of detection, but are key contributors to overall flavor and mouthfeel. Many are derived from the interactions between yeast enzymes and plant-derived compounds, modifications that alter the organoleptic properties of flavor molecules, including the threshold of detection, and have a significant effect on flavor profiles. Engineered strains are able to modulate the concentrations and the ratios of grape berry and hop-derived compounds, and some work has also been done on *de novo* biosynthesis of flavors.

The molecules known as terpenoids are important odorants in wine and beer. The monoterpenes (built from ten carbons), sesquiterpenes (built from fifteen carbons), and their derivatives, including alcohols, esters, oxides, and epoxides are key contributors to grape berry and hop aroma and the aroma of the final fermentation product. A wine yeast capable of biosynthesizing grape terpenes *de novo* was generated by introducing a linalool synthase from *Clarkia breweri*, regulated by the high-strength *TDH3* promoter¹⁰. Microvinification experiments revealed that linalool production peaked within two days and that other than linalool production, no other significant changes were found in the wine’s volatile profile compared to wine made with a control strain. Later work from the same group engineered the same strain to express a geraniol synthase from *Ocimum basilicum*. It was found that native yeast enzymes generated a variety of other terpene derivatives from geraniol, including other monoterpenes and esters¹¹. In both studies, however, the synthases were expressed from high-copy plasmids, which are unstable and require selective media for propagation, and are therefore impractical for industrial applications.

Another approach for increasing final terpene content is to express an enzyme that cleaves glycosidic linkages: in plants, a large proportion of total terpenes (and other aromatic compounds) are found as odorless precursors conjugated to sugars. Hydrolysis of the glycoside conjugate by enzymes such as β -D-glucosidases, α -L-arabinofuranosidases, and α -L-rhamnosidases releases the flavor compound and enhances the aroma of wine or beer¹². Expression of an α -L-rhamnosidase from *Aspergillus aculeatus* and a β -glucosidase from *Candida molischiana* in industrial wine strains resulted in increased linalool, α -terpineol, and nerol content¹². Many non-*Saccharomyces* species also show a range of glycosidase activity. Twelve oenological isolates were found to exhibit β -glucosidase, β -xylosidase, and α -rhamnosidase activity, potential targets for engineering aromatic compound release in industrial wine strains¹³.

During fermentation, many aromatic molecules imparted by grapes or hops are further functionalized by modifying enzymes native to yeast. The oxidoreductase encoded by *OYE2* reduces geraniol into citronellol¹⁴. While geraniol imparts rose-like,

floral, and citrus aromas, citronellol imparts orange blossom aromas. The alcohol acetyltransferases encoded by *ATF1* and *ATF2* are responsible for volatile acetate ester and terpenyl acetate production¹⁵. Engineered strains overexpressing *ATF1* and *ATF2* produced significantly higher concentrations of the esters ethyl acetate, isoamyl acetate, 2-phenylethyl acetate, and ethyl caproate compared to control strains¹⁶. Wines made with these strains produced a heavier fruity aroma, including peach, apricot, apple, and banana aromas. Conversely, overexpression of the esterase encoded by *IAH1* decreased the production of these esters. Deletion of both *ATF1* and *ATF2* abolished isoamyl acetate production in a commercial brewing strain; however, other esters were still produced, demonstrating the existence of other ester-forming enzymes. Overexpression of *ATF1* in an industrial lager yeast increased isoamyl acetate production 180-fold and ethyl acetate production 30-fold; overexpression of *ATF2* increased isoamyl acetate 10- to 25-fold (depending on the allele expressed) and ethyl acetate 2- to 3-fold¹⁵. This work opens up possibilities for developing new yeast strains with improved ester aroma production and engineering better control over ester synthesis during industrial fermentations.

Another group of flavor molecules found in both grapes and hops, the volatile thiols, are also modified by yeast during fermentation. These sulfur-containing compounds are found at very low concentrations in both plants; however, due to their extremely low sensory perception threshold, they make an important contribution to wine (especially Sauvignon blanc) and beer aroma by conferring “tropical” characters. Similar to many terpenes, they are generally found as odorless precursors conjugated to sugars and are released as aromatic compounds by yeast enzymes during fermentation. The most important volatile thiols are 4-mercapto-4-methylpentan-2-one (4MMP, box tree and passionfruit aromas) and 3-mercaptohexan-1-ol and 3-mercaptohexylacetate (3MH and 3MHA, passionfruit, grapefruit, gooseberry, and guava aromas). Overexpression of the yeast beta-lyase gene *STR3* in commercial wine strain VIN13 led to an increase in 3MH release¹⁷. In fermentations with Sauvignon blanc grape must, the engineered strain released 9.5 times more 3MH than a control strain. Expression of *tnaA* from *E. coli*, encoding a tryptophanase with cysteine beta-lyase activity, led to an increase, up to 25-fold, in both 3MH and 4MMP release¹⁸. Wines made with these strains exhibited intense passionfruit aromas. Overexpression of *ATF1* in commercial wine strain VIN13 led to increased conversion of 3MH into 3MHA, demonstrating the involvement of Atf1p in both ester and thiol aroma modulation.

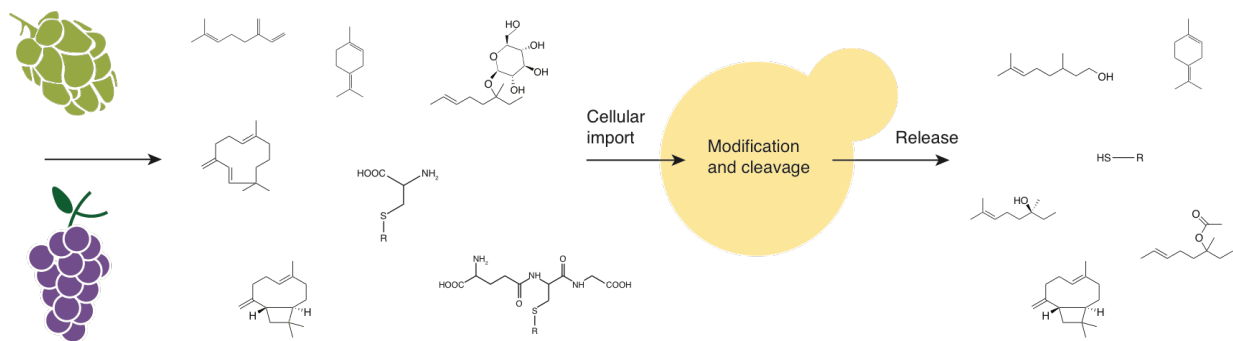


Figure 1.2. Aromatic flavor molecules made by hops and grapes. Odorants and odorant precursors include monoterpenes, sesquiterpenes, terpene glycosides, and conjugated thiols. Some remain unaltered in the final product; others are imported into yeast and modified before release, such as by reduction, cleavage of the conjugated molecule, and acetylation.

Even though plant flavor profiles are complex mixtures of hundreds of aromatic compounds, certain molecules are known to be primarily responsible for specific flavors, including vanillin for vanilla, nootkatone for grapefruit, and 3-isobutyl-2-methoxypyrazine for green bell peppers. Recent work showcased the potential of introducing biosynthetic pathways for plant-derived compounds into industrial yeast in order to impart specialty flavors to fermentation products. Introduction of four genes from the phenylpropanoid pathway into a wine yeast strain produced detectable amounts (well above the sensory threshold) of raspberry ketone, the compound responsible for raspberry aroma, in fermentations with Chardonnay grape juice¹⁹.

Several metabolic byproducts produced by yeast during fermentation impart undesirable flavor and aroma characteristics and must be removed from the fermentation. Some of these compounds - the vicinal diketones (butanedione, also known as diacetyl, and pentanedione), organic acids, and phenolics - are occasionally desired in specific styles (diacetyl in Chardonnay and certain British ales, for example) but are otherwise, and at high concentrations always, considered off-flavors. Others, such as hydrogen sulfide in wine, are never desired at any concentration.

The vicinal diketones (VDKs) are formed by the breakdown of other fermentation byproducts and are typically removed from beer by a resting period after fermentation, during which they are absorbed and metabolized by the yeast. Diacetyl is reduced into flavorless acetoin and may be further reduced into 2,3-butanediol; pentanedione is reduced into 2,3-pentanedione. This resting period increases the fermentation time by several weeks. Early work introduced an acetolactate decarboxylase into the genome of a brewing yeast strain, conferring the ability to convert α -acetolactate (the diacetyl precursor) directly into acetoin, thereby bypassing diacetyl formation²⁰. *ILV5*, encoding an acetohydroxyacid reductoisomerase normally expressed in the mitochondria, is also capable of metabolizing the available α -acetolactate. Expression of an N-terminal truncated form localized to the cytosol in a high VDK-producing lager strain decreased total VDK production by two-thirds and shortened the time required to decrease VDKs to levels below their threshold of detection²¹. Replacement of *ILV2* (encoding acetolactate synthase) with the full-length *ILV5* in a lager strain resulted in a 60%

decrease in VDK production²². Similarly, deletion of two copies of *ILV6*, encoding the regulatory subunit of acetolactate synthase, in a commercial lager strain gave a similar decrease in diacetyl concentrations (65%)²³. Recent work combined these approaches - deletion of *ILV2*, expression of *ILV5* - with the overexpression of *BDH1* (butanediol dehydrogenase) or *BDH2* (a putative dehydrogenase with similarity to *BDH1*). Addition of *BDH1* or *BDH2* had only a modest effect on the decrease in diacetyl concentrations, however^{24,25}.

High concentrations of the fermentation byproduct acetic acid add an undesired vinegar flavor to wine. Introduction of a mutated version of *YAP1*, encoding a basic leucine zipper transcription factor involved in oxidative stress tolerance, led to a 90% reduction in acetic acid production. Four strains were isolated with heterozygous mutations in *YAP1*, all of which caused a premature truncation of the protein, but only one strain retained wild-type fermentation capacities²⁶. Deletion of *AAF1*, encoding a zinc-finger transcription factor that regulates acetate production, reduced acetic acid production by as much as 39.2% without increasing acetaldehyde concentrations²⁷. Deletion of the *AAF1* target genes *ALD4* and *ALD6* in the *AAF1* deletion background resulted in growth defects; deletion of *AAF1* paralog *RSF2* had no effect on growth. While the four genes lack synergistic roles in acetic acid production regulation, deletion of *AAF1* and deletion of *ALD6* (independently), led to large decreases in acetic acid production. Conversely, overexpression of *AAF1* increased acetic acid levels in wild-type and *ALD4* deletion backgrounds but, interestingly, the opposite effect was observed in an *ALD6* deletion background.

1.5. Academic examples: fermentation characteristics

Flocculation is the reversible aggregation of yeast cells into multicellular flocs, which is accompanied by their separation from the medium (either by rising to the top or falling to the bottom). This process is driven by the expression of flocculation (*FLO*) genes that encode surface-displayed proteins that bind to the cell walls of neighboring yeast cells. Brewing and wine yeasts have evolved over hundreds of years to exhibit specific flocculation phenotypes. Wild yeasts generally do not flocculate whereas domesticated strains exhibit a range of flocculation phenotypes. Generally, brewer's yeast begins flocculation at the end of fermentation, thereby clarifying the beer and allowing brewers to easily collect the yeast. However, many brewers use non-flocculating strains or low flocculating yeast, removing them via centrifugation or filtration, or strains that flocculate in response to a lowering of the temperature (cold crash). Flocculation dramatically reduces the nutrient availability for individual yeast cells, thereby inhibiting their metabolic and fermentation capabilities; because of this, strains that flocculate prematurely lead to incomplete fermentations and therefore, the timing of flocculation is key.

Expression of the flocculation gene *FLO1* during late fermentation by replacing the wild-type *FLO1* promoter with the stationary phase-induced *HSP30* promoter caused a nonflocculent strain to flocculate toward the end of fermentation²⁸. Similarly, flocculation genes *FLO1*, *FLO5*, and *FLO11* were placed under the regulation of the promoters *ADH2* and *HSP30* in a lab strain. Each strain exhibited specific flocculation characteristics regarding timing and intensity: *FLO1* strains showed the strongest

flocculation, *FLO11* the weakest flocculation but the strongest flor (surface film) formation; maximum flocculation was observed approximately six hours after glucose exhaustion with the *ALD2* promoter, for *HSP30* it peaked at the time of glucose depletion; overall, the *ALD2* promoter induced stronger flocculation than the *HSP30* promoter²⁹. Similar results were found in wine strains BM45 and VIN13; however, *FLO5* transformants were more flocculent than the corresponding *FLO1* strains³⁰. While *FLO11* strains did not flocculate in standard laboratory media or synthetic grape must fermentation conditions, they were found to strongly flocculate in authentic red wine-making conditions. Interestingly, this flocculation phenotype seems to be due to a different mechanism than the canonical *FLO1* and *FLO5*-mediated flocculation³¹.

During fermentation, yeast import and metabolize certain available sugars, known as fermentable sugars, producing ethanol and releasing carbon dioxide. In grapes, the most abundant fermentable sugars are glucose and fructose, in malt the most abundant is maltose, followed by maltotriose and glucose. Yeast consume certain sugars preferentially, and all strains prioritize glucose. Total sugar consumption is, however, strain dependent: brewing strains exhibit varying abilities to consume maltotriose and wine strains fructose, resulting in a range of ethanol concentrations and leftover (residual) sugar³². The ability of a given strain to consume the available sugars is called attenuation; strains range from strong attenuators (those that consume all fermentable sugars) to low attenuators, although most commonly used strains are medium attenuators at least, leaving only a small percentage of the total fermentable sugars.

Maltotriose is used only in the later stages of brewing fermentations and incomplete maltotriose consumption is a common problem, especially in high-gravity brewing. (Gravity is a measurement of the total solid content dissolved in the wort, including the fermentable sugars; high-gravity brewing refers to the high-gravity of the wort pre-fermentation, resulting in high alcohol percentage beers). Introduction of a maltotriose transporter, encoded by *MTT1*, into a lager strain resulted in a 17% increase in maltotriose uptake. An altered version of *MTT1* (with amino acid replacements at the C-terminal end) further increased uptake by 105%³³. Using a different approach, a maltase encoded by *MAL32* was targeted to the cell surface, bypassing maltotriose transport by allowing for extracellular hydrolysis of maltotriose. The maltase was found to be present during fermentation and engineered strains used maltotriose and maltose faster than control strains³⁴.

Non-fermentable sugars, also present in grape must and wort, are not metabolized by standard brewing and wine yeasts but instead contribute to the body and mouthfeel of the final product. Approximately 25% of the sugars in wort are non-fermentable, consisting of longer polysaccharides known as dextrans and monosaccharides such as ribose, arabinose, and xylose^{34,35}. *Saccharomyces cerevisiae* var. *diastaticus* is a strain variant capable of metabolizing dextrans by secreting a glucoamylase enzyme that breaks down 1,4- α linkages. Because of this ability, *diastaticus* is classified as a superattenuator and a contaminant, leading to secondary fermentations that excessively over-carbonate the resulting beer³⁶. Three genes in *S. cerevisiae* var. *diastaticus*, *STA1*, *STA2*, and *STA3*, encode the three extracellular

glucoamylase isozymes. Brewing strains have been engineered to express *STA1* and *STA2*; in all cases, fermentations conducted with these strains resulted in higher sugar consumption compared to parent strains^{37–39}. A brewing yeast expressing *STA1* was also constructed by mating. Again, a larger percentage of available sugars were consumed by this strain, resulting in a beer with a higher ethanol concentration³⁵.

The final ethanol content of wine and beer varies depending on the attenuation capability of each strain but in beer typically ranges from 3–8%⁴⁰ and in wine 12–15%⁴¹. Higher attenuation results in a drier product with a higher ethanol content. Recently, consumer demand has shifted towards lower alcohol products that still contain the same full flavor profiles. Several strategies exist for directing carbon away from ethanol formation and towards other products. A large number of modifications predicted to impact ethanol production resulted in a strain with increased *GPD1* expression, encoding glycerol-3-phosphate dehydrogenase⁴². This strain gave decreased ethanol yields balanced by increased glycerol yields. Increasing the copy number of the overexpressed *GPD1* further decreased ethanol production. However, the strains also produced increased concentrations of acetaldehyde and acetoin, requiring further engineering to overcome these negative effects. Screening of 66 strains with deletions in genes encoding enzymes involved in central carbohydrate metabolism resulted in two strains with lower ethanol yields. Based on these results, expression of the *TPS1* gene (the synthase subunit of trehalose-6-phosphate synthase/phosphatase complex) was slightly upregulated, resulting in a strain with decreased ethanol yields balanced by increased trehalose accumulation, a reserve carbohydrate⁴³. Expression of the *Aspergillus niger* gene encoding glucose oxidase (GOX) reduced alcohol production by 2% in microvinification experiments with a lab yeast strain. GOX is secreted into the culture medium and converts glucose into hydrogen peroxide, D-glucono-1,5-lactone, and gluconic acid, lowering the fermentable sugar content available to the strain. However, further research is required to ensure the safety and sensory qualities of the wine are not impacted by such a strain⁴⁴.

1.6. Conclusions

Customization of brewing and wine yeasts through metabolic engineering, the newest update to the centuries old beer and wine industries, will increase the variety of strains available to the brewer and winemaker. There is huge potential in the production of flavors (including specialty plant flavors) and the improvement of fermentation performance characteristics. A lot of progress has been made, especially in the wine industry, where engineered commercial strains are already available. However, almost all academic work to date relies on the use of high-copy plasmids with selectable markers, which are unstable and require selective media for propagation, and are therefore impractical for industrial applications. In addition, much of the early proof-of-concept work was carried out in lab yeast strains and needs to be translated to industrial strains, while work done in industrial strains needs to be confirmed in industrial scale fermentations.

2. Chapter 2 - Industrial brewing yeast engineered for the production of primary flavor determinants in hopped beer

Including material from submitted work: Denby, C.M., **Li, R.A.**, Vu, V.T., Costello, Z., Lin, W., Chan, L.J.G., Williams, J., Donaldson, B., Bamforth, C.W., Petzold, C.J., Scheller, H.V., Martin, H.G., & Keasling, J.D. (2018).

2.1. Abstract

Flowers of the hop plant provide both bitterness and “hoppy” flavor to beer. Hops are, however, both a water and energy intensive crop and vary considerably in essential oil content, making it challenging to achieve a consistent hoppy taste in beer. Here we report that brewer’s yeast can be engineered to biosynthesize aromatic monoterpene molecules that impart hoppy flavor to beer by incorporating recombinant DNA derived from yeast, mint, and basil. Whereas metabolic engineering of biosynthetic pathways is commonly enlisted to maximize product titers, tuning expression of pathway enzymes to affect target production levels of multiple commercially important metabolites without major collateral metabolic changes represents a unique challenge. By applying state-of-the-art engineering techniques and a framework to guide iterative improvement, strains are generated with target performance characteristics. Beers produced using these strains are perceived as hoppier than traditionally hopped beers by a sensory panel in a double-blind tasting.

2.2. The brewing process

The four ingredients essential to the brewing process are water, malt, hops, and yeast. Malt is cereal grains (such as barley) which have been germinated and kilned to release stored starches. The female flowers of the hop plant, *Humulus lupulus* L., are added at various stages in the brewing process for their resin, which imparts bitter flavors to beer, and essential oil, which imparts floral/citrus hoppy aromas to beer. Brewing begins with the mixing of malt and hot water, a process known as mashing, which breaks down the starches into fermentable sugars that yeast can utilize. The resulting sugar-rich solution, called wort, is moved to a kettle and boiled with additional ingredients. Bittering and aroma hops can both be added at this step, but aroma hops are typically added later in the boil to avoid evaporation of the aromatic compounds. The wort is then cooled, and large solid particles removed, and yeast is added to begin fermentation. Hops may be added again towards the end of fermentation, a process known as dry hopping, to impart a fuller, hoppier aroma. Once fermentation (and optional dry hopping) is complete, the beer is conditioned with a resting period that removes unwanted flavors and by-products, filtered, and carbonated.

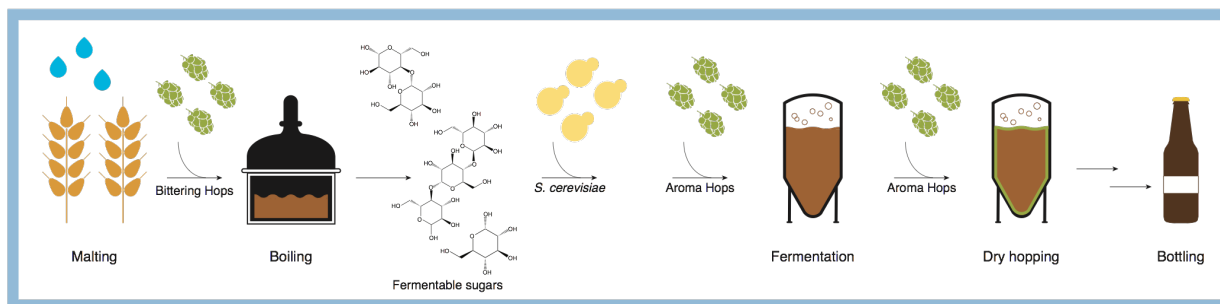


Figure 2.1. Overview of the brewing process.

2.3. Introduction

During the brewing process, *Saccharomyces cerevisiae* converts the fermentable sugars from grains into ethanol and a host of other flavor-determining by-products. Flowers of the hop plant, *Humulus lupulus* L., are typically added during the wort boil to impart bitter flavor and immediately before or during the fermentation to impart “hoppy” flavor and fragrance (**Figure 2.2**). Over the last two decades, consumers have displayed an increasing preference for beers that contain hoppy flavor. Hops are an expensive ingredient for breweries to source (total domestic sales have tripled over the last 10 years due to heightened demand) and a crop that requires a large amount of natural resources: ~100 billion L of water is required for annual irrigation of domestic hops and considerable infrastructure is required to deliver water from its source to the farm^{45,46}. Further, hops vary considerably in essential oil content, making it challenging to achieve a consistent hoppy taste in beer.

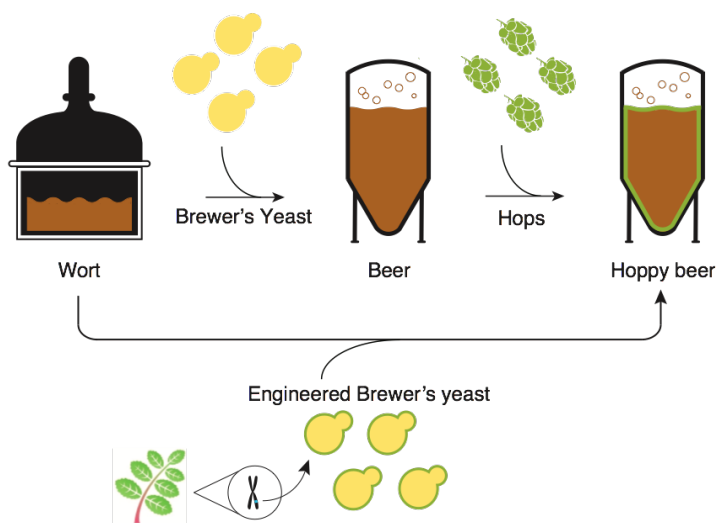


Figure 2.2. Engineering brewer's yeast to express the monoterpene biosynthetic pathway to replace flavor hop addition. During the brewing process, *S. cerevisiae* converts wort—a barley-extract solution rich in fermentable sugars—into ethanol and other by-products. Hops are added immediately before, during, or after fermentation to impart “hoppy” flavor. Engineered strains produce linalool and geraniol, primary flavor components of hoppy beer, thereby replacing hop additions.

Hop flowers are densely covered by glandular trichomes, specialized structures that secrete secondary metabolites into epidermal outgrowths⁴⁷. These secretions accumulate as essential oil, which is rich in various terpenes, the class of metabolites that impart hoppy flavor to beer. Considerable research has investigated which of these molecules are primarily responsible for this flavor⁴⁸; these studies are complicated by genetic, environmental, and process-level variation⁴⁹ and have suggested that the bouquet of flavor molecules contributed to beer by hops is complex. Nonetheless, the two monoterpene molecules linalool and geraniol have been identified as primary flavor determinants by several sensory analyses of hop extract aroma^{50–52} and finished beer taste and aroma^{51,53–55} and together, they are major drivers of the floral aroma of Cascade hops⁵³, the most widely used hop in American craft brewing⁵⁶. Previous metabolic engineering efforts have achieved microbial monoterpene biosynthesis in various microbial hosts. Work in a domesticated wine yeast has demonstrated the feasibility of producing monoterpene compounds by biosynthesis in yeast by overexpression of a geraniol synthase from a high-copy plasmid propagated in selective media⁵⁷. However, engineering genetically stable, controlled, precise production of a combination of specific flavorants in any industrial food-processing agent has remained a formidable challenge.

In this work, we create drop-in brewer's yeast strains capable of biosynthesizing monoterpenes that give rise to hoppy flavor in finished beer, without addition of flavor hops. To achieve this end, we identify genes suitable for monoterpene biosynthesis in yeast; we develop methods to overcome the difficulties associated with stable integration of large constructs in industrial strains; we adapt genetic tools to generate a collection of engineered industrial yeast strains on an unprecedented scale; we develop computational methods to affect precise biosynthetic control and leverage them to create an iterative framework towards target production levels. Ultimately, sensory analysis performed with beer brewed in pilot industrial fermentations demonstrates that engineered strains confer hoppy flavor to finished beer.

2.4. Results

The monoterpene synthases that catalyze the reaction of geranyl pyrophosphate (GPP) to linalool and geraniol in hops have not yet been identified⁵⁸. However, genes from other plant species have been shown to encode these activities. To identify a linalool synthase (LIS) gene for functional heterologous expression in yeast, we expressed six different plant-derived LIS genes in a lab yeast strain engineered for high GPP precursor supply (**Figure 2.3a**). However, none of the full-length proteins exhibited sufficient activity to achieve target monoterpene concentrations in finished beer (**Figure 2.3d**). In plants, monoterpenes are biosynthesized in chloroplasts; plant monoterpene synthases, therefore, typically contain an N-terminal plastid targeting sequence (PTS) composed of 20-80 polar amino acids, which is cleaved to produce a mature protein. Truncation of the PTS sequence can improve expression and activity of microbially expressed monoterpene synthases^{59,60}. However, predicting portability of enzymes from plant sources to yeast and identifying the optimal truncation site is challenging. We therefore screened candidate LIS variants from different sources and with different truncations sites for increased activity (**Figure 2.3**). We tested bioinformatically-

predicted⁶¹ PTS sites and observed a substantial increase in activity for the *Lycopersicon esculentum* LIS (**Figure 2.3d**). We also used a heuristic, structure-based approach for PTS prediction: a double arginine (RR) motif that functions as part of an active-site lid preventing water access to the carbocationic reaction intermediate⁶² and lies immediately C-terminal to the PTS in several well-characterized terpene synthases^{59,60}. We observed the highest linalool titer from the truncated *M. citrata* LIS (t67-McLIS). An analogous screen of six geraniol synthases revealed that the heterologous expression of the full-length protein from *O. basilicum* (ObGES) leads to geraniol production in yeast (**Figure 2.3d**).

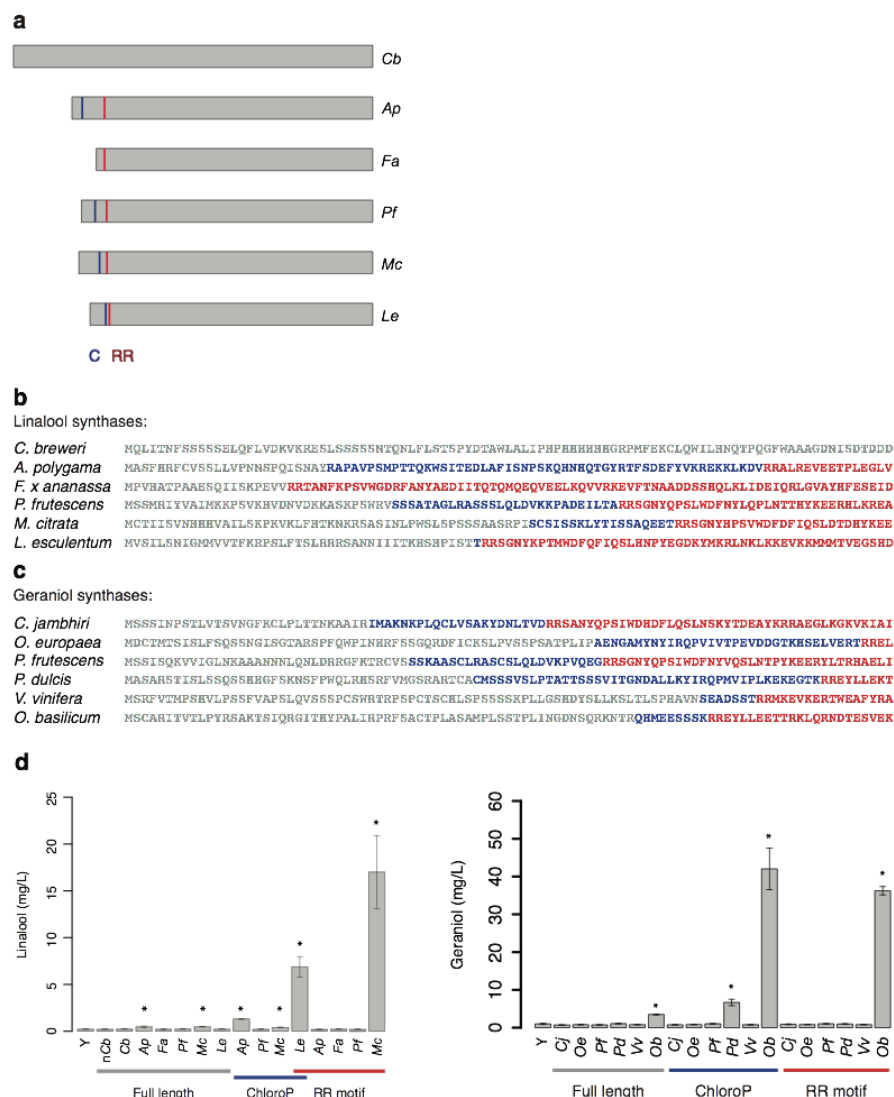


Figure 2.3. a Six full-length plant-derived linalool synthase genes, as well as PTS-truncated variants, were expressed on high copy plasmids. Full-length genes and PTS-truncated genes predicted by either ChloroP (C) or the RR-heuristic method (RR) are indicated by colored lines. **b,c** Sequences of N-terminal amino acids for linalool and geraniol synthases, respectively. Red text indicates the PTS-truncated protein sequence as predicted by the RR-heuristic method, blue text indicates additional sequence predicted by ChloroP, and gray text indicates additional sequence corresponding to the full-length peptide. The linalool synthase from *C. breweri* does not contain a RR motif and is not predicted to contain a PTS by ChloroP. **d** Linalool and geraniol titers produced by cells overexpressing monoterpene synthase

genes (expressed from high-copy plasmids) as measured by GC/MS. Plant species of origin are indicated by initials (*Citrus jambhiri*, Cj; *Olea europaea*, Oe; *Perilla frutescens*, Pf; *Phyla dulcis*, Pd; *Vitis vinifera*, Vv; *Ocimum basilicum*, Ob), Y indicates the control strain, full-length peptides and PTS-truncated peptides predicted by either ChloroP or the RR-heuristic method are indicated by colored bars underlining the plots: grey, blue and red, respectively. Error bars correspond to mean $\pm$ standard deviation of three biological replicates. Asterisks indicate statistically significant increases in monoterpene production compared with the control strain (Y) as determined by a *t*-test using p-value < 0.025. The LIS from the California wildflower *Clarkia breweri* has been shown to increase production of linalool when heterologously expressed in plants⁶³ and in yeast⁶⁴. However, when *C. breweri* LIS was expressed, either with native codons (nC*b*) or “yeast-optimized” codons (C*b*), linalool was not detected. The *Mentha citrata* LIS (M*c*) truncated at the RR motif was identified as sufficiently active to allow for monoterpene production at levels characteristic of commercial beer and was chosen for integration into brewer’s yeast strains. The full-length *O. basilicum* geraniol synthase (O*b*) was identified as sufficiently active to allow for monoterpene production at levels characteristic of commercial beer, with the truncated peptide predicted by either method resulting in a 10-fold increase in production. Note that this geraniol synthase has been used in previous metabolic engineering efforts.

Once we identified monoterpene synthases that were sufficiently active in *S. cerevisiae*, we set out to engineer brewer’s yeast strains capable of producing monoterpenes during beer fermentation. Yeast naturally produces the sesquiterpene precursor farnesyl pyrophosphate (FPP) through the ergosterol biosynthesis pathway, as FPP serves as a precursor for essential metabolites including hemes and sterols. While the flux through this pathway is tightly regulated, extensive metabolic engineering efforts have informed key genetic modifications that obviate regulatory checkpoints^{65,66} and increase monoterpene precursor supply⁶⁷ (**Figure 2.4**). HMG-CoA reductase (HMGR) is one of the key rate-limiting steps of the pathway and is controlled by an inhibitory regulatory domain that responds to product accumulation⁶⁸. Overexpression of a truncated form of yeast HMGR lacking the regulatory domain (tHMGR) results in increased flux towards end-products⁶⁹. A downstream enzyme, FPP synthase (FPPS), catalyzes the sequential condensation of two isopentenyl pyrophosphate (IPP) molecules with dimethylallyl pyrophosphate (DMAPP). GPP, the immediate precursor of monoterpene biosynthesis, is formed as an intermediate of the sequential reactions. While the high processivity of the wild-type FPPS limits the intracellular availability of GPP, a mutant (FPPS*) has been identified that reduces processivity, thereby increasing production of GPP-derived end products⁷⁰. Based on these observations, we hypothesized that modulating the expression of tHMGR, FPPS*, t67-McLIS, and ObGES would result in brewer’s yeast strains capable of producing linalool and geraniol during fermentation at concentrations encompassing those typical of finished beer (~0.2 mg/L)^{53,71}.

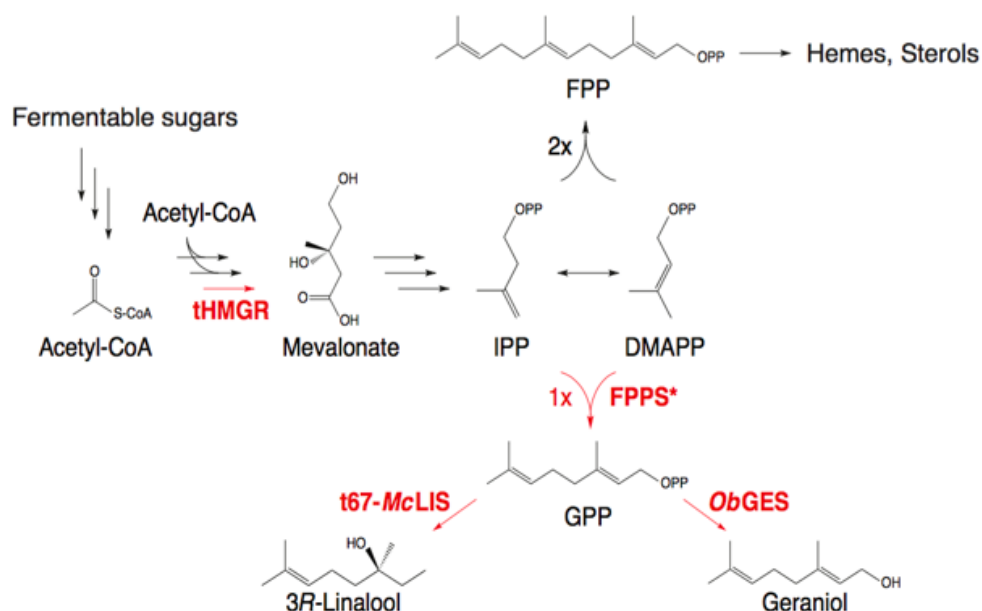


Figure 2.4. Schematic of genetic modifications incorporated into brewer's yeast strains. Red arrows correspond to genes that were modulated to tune linalool/geraniol production. Details on genetic modifications, strain construction procedure, and constructs used to incorporate genetic modifications can be found in Methods and Supplementary Tables 7-9.

In devising a strategy to modulate pathway activity, two challenges were considered. First, *de novo* design and generation of a collection of multi-gene constructs is difficult, time-consuming, and expensive. To circumvent this challenge, we combined an existing toolkit of yeast genetic parts with a Golden Gate assembly strategy for facile design and rapid pathway construction⁷² (**Figure 2.5a, d**). Second, incorporating large (i.e. > 10kb) genetically stable DNA constructs into brewer's yeast has not been reported, and is complicated by their ploidy as well as concerns regarding the incorporation of selection markers in food-processing agents. We therefore developed a Cas9-mediated methodology for stable and marker-less pathway integration (**Figure 2.5a-c**). Our method leverages a colorimetric assay to screen for positive transformants and allows for macroscopic visualization of successful integration events. Interestingly, this method also allowed us to visualize the high degree of genetic instability associated with single-locus integration (**Figure 2.5b,c**). By combining the assembly and integration strategies, we were able to generate strains with a diverse set of genetic designs, where each strain contained a unique combination of promoters driving expression of the four modulated genes (**Figure 2.5d**).

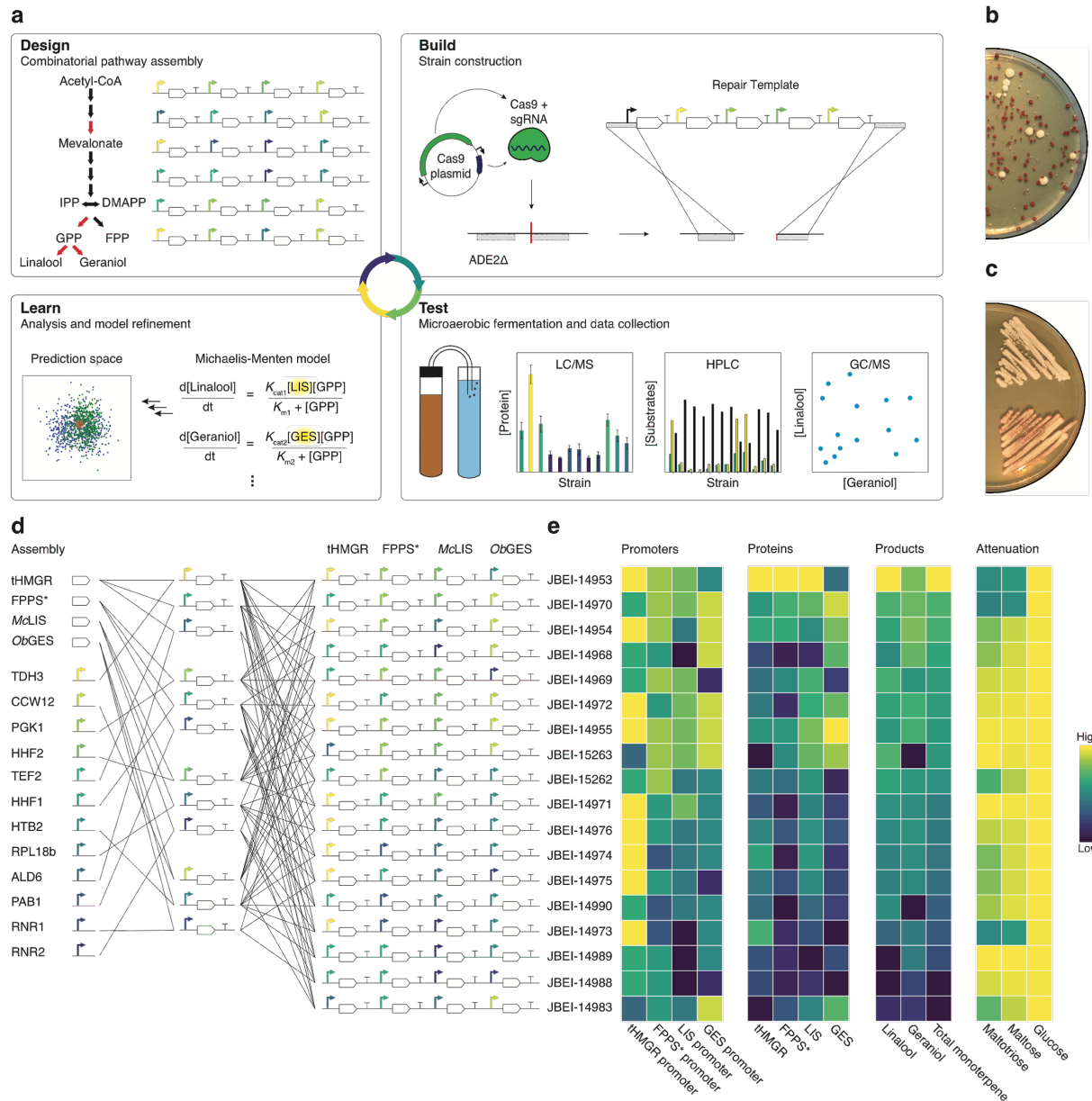


Figure 2.5. Iterative improvement of strain design towards targeted monoterpene production levels. **a** Schematic of design-build-test-learn cycle. Design: constructs were designed by combining yeast toolkit parts (i.e. promoters, terminators, linkers, etc.) with monoterpene biosynthesis pathway genes. Build: methodology for integrating constructs into brewer's yeast. Note that, for simplicity, only a single allelic copy of the ADE2 locus is diagrammed. The ADE2Δ strain was co-transformed with a Cas9/sgrRNA plasmid and repair template, which targeted a double-stranded break (DSB) in the ADE2 3' sequence. Test: data were collected using LC/MS, HPLC, and GC/MS. Learn: Correlation analyses informed design principles. Mathematical models were used to evaluate the extent to which design principles improved strain search efficiency. Variables corresponding to measured protein levels are highlighted. **b,c** Transformation plate illustrating colorimetric screening method. ADE2 encodes an enzymatic step in purine biosynthesis and its deletion results in accumulation of a metabolite with red pigment when grown on media containing intermediate adenine concentration. Because the repair template contains the ADE2 gene, templated DSB repair results in a white colony phenotype. Because brewer's yeasts have multiple allelic copies of ADE2, stable integration requires repair at multiple ADE2Δ genomic loci. White colonies streaked from transformation plates result in either white colony color (**b**) or variegated colony color (**c**); White colony color corresponds to homozygous integration; variegated colony color corresponds to

heterozygous integration, illustrating genetic instability of heterozygous allele containing a large DNA construct. **d** Illustration of assembly steps from parts (promoters/genes) to gene cassettes, to repair templates for first iteration strains. **e** Relative promoter strengths with corresponding protein and product abundances and sugar consumption (attenuation). Strains are sorted by total monoterpene production.

In preliminary experiments the rate of stable pathway integration was considerably lower than that previously reported for gene deletions in other industrial yeast strains^{73,74}. This could be explained by methodological differences or by the extended repair template size. To assist with the challenge of identifying stable pathway transformants, the integration strategy leverages a colorimetric assay commonly used in yeast genetic screens^{75,76}. That is, a yeast strain lacking the ADE2 gene accumulates a red intermediate derived from the adenine biosynthesis pathway. For the strain construction strategy, an *ade2* deletion strain was generated from the parent strain and a copy of the ADE2 gene was included in the repair template as part of the 5' homology arm (**Figure 2.5a**). Therefore, transformants with on-target pathway integration appeared white on a transformation plate, while unsuccessful transformants remained red (**Figure 2.5b**). In order to confirm that all genetic copies of the ADE2 locus were successfully replaced with the biosynthetic pathway gene construct, integration events were confirmed by diagnostic PCR. Results revealed that a fraction of the white transformants were heterozygous for the biosynthetic pathway genes at the ADE2 locus. Interestingly, the heterozygous colonies exhibited a variegated phenotype upon further propagation (**Figure 2.5c**). This serendipitous finding not only illustrates the instability of heterozygote genetic modifications in industrial strains, but also highlights a feature of the colorimetric assay that can be used to distinguish unstable heterozygous transformants from stable homozygous transformants. Notably, when red colonies from the variegated patch were restreaked, resulting colonies remained red, but when white colonies from the variegated patch were restreaked, resulting colonies exhibited the variegated phenotype (data not shown). Thus the heterozygous strains exhibit a high rate of loss of heterozygosity (LOH), and that LOH mutants were biased towards loss of heterologous pathway genes, rather than gain of heterologous pathway genes.

Without empirical data, it is difficult to predict the relationship between specific genetic designs and metabolic end-product concentrations^{77,78}. To improve search efficiency towards desired monoterpene concentrations, we separated our design-build-test process (**Figure 2.5a**) into two stages, thereby affording us an opportunity to first sample a small subset of design space and then hone subsequent designs towards desired production profiles. An initial set of 18 strains containing promoters predicted to span a wide range of expression strengths were constructed and grown under microaerobic fermentation conditions that mimicked an industrial brewing process. We found that these first iterations strains produced monoterpenes within the range of commercial concentrations, though were generally lower (**Figure 2.6**).

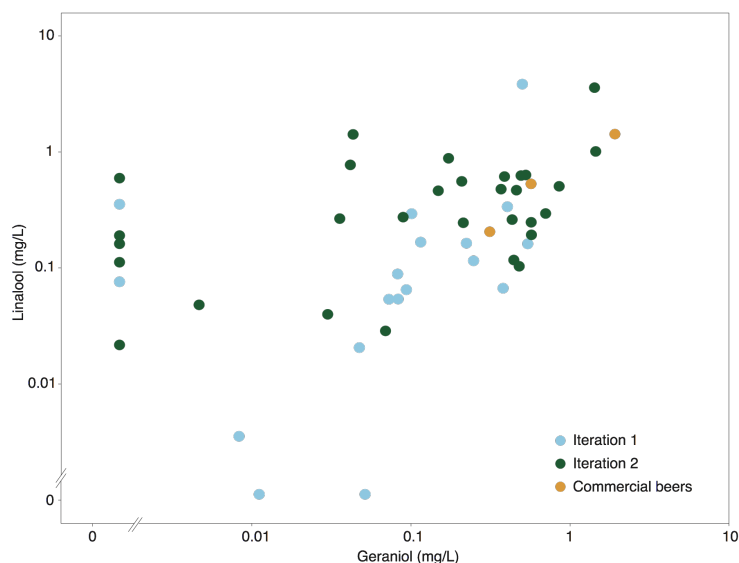


Figure 2.6. Production of monoterpenes by engineered strains. Linalool and geraniol production of engineered yeast strains compared to commercial beers, plotted in $\log_{10}$ space. For relationships between flavor determinant concentration and taste intensity, the logarithm of a stimulus is typically proportional to the logarithm of the perceived intensity, such that the distance between points in $\log_{10}$ space is expected to be directly proportional to the magnitude of taste difference. First and second iteration points represent the mean of three biological replicates. Standard deviation values are listed in Supplementary Table 10. In ascending order of monoterpene concentration, commercial beers are Pale Ale, Torpedo Extra IPA, and Hop Hunter IPA, from the Sierra Nevada Brewing Company.

Some strains exhibited a reduced fermentation capacity (**Figure 2.7**) including the strains closest to commercial concentrations. However, reduced fermentation capacity did not correlate with monoterpene production, suggesting that the fermentation defects were not primarily due to monoterpene toxicity.

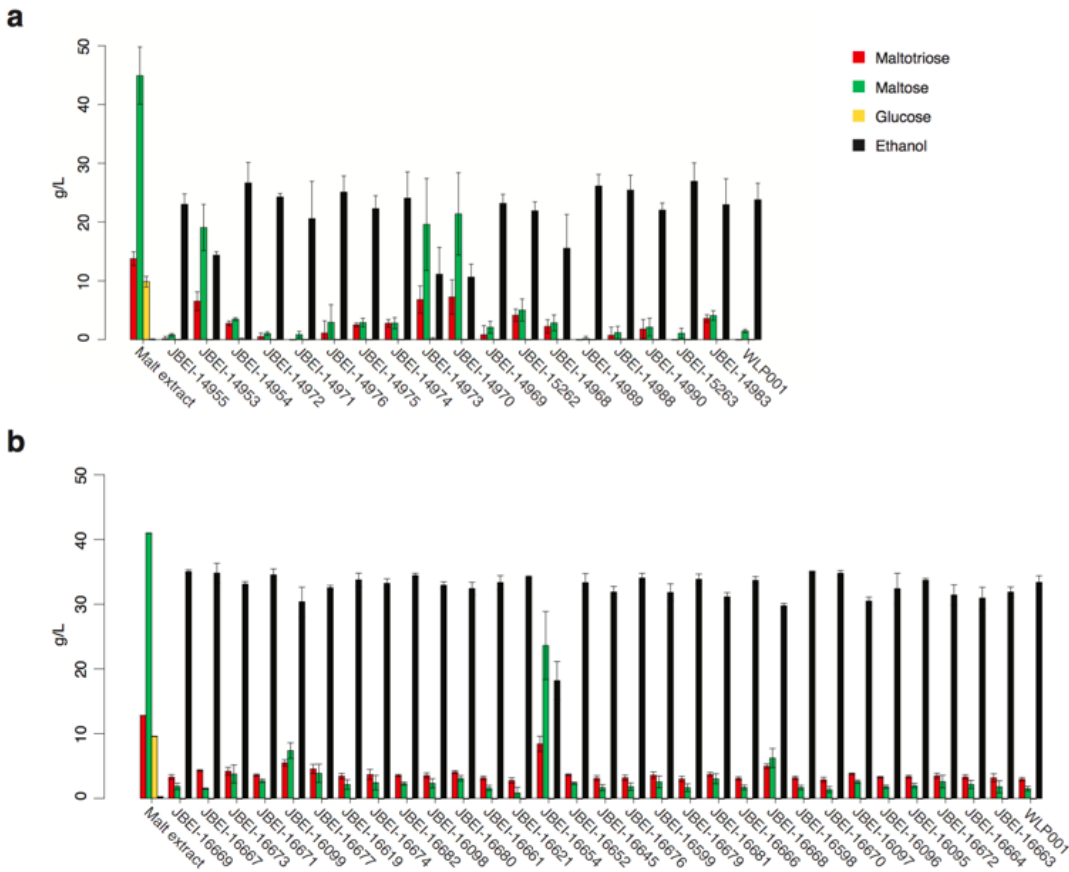


Figure 2.7. Fermentable sugars and ethanol remaining/produced after fermentation by engineered brewing strains from first (a) and second iteration strains (b) compared to parent strain *WLP001* and unfermented medium. Error bars correspond to mean $\pm$ standard deviation of three biological replicates.

There are several potential explanations for this observation based on previous studies. Firstly, heterologous protein expression can introduce a metabolic burden by overloading DNA replication machinery and/or protein expression machinery^{77,79}. This is not a likely explanation in the present study. The integrated DNA amounts to a very small percentage of the total genome size ($\sim 0.1\%$) and the heterologous protein expression is small relative to the expression of the total proteome ($\sim 0.03\%$ – 0.09% of the total proteome). In addition, no significant correlation was observed between relative heterologous protein expression and remaining fermentable sugar (correlation of 0.27, p -value of 0.02). Finally, we measured the rate of sugar consumption over a 5-day fermentation for the strain predicted to drive highest expression of heterologous proteins and observed indistinguishable growth properties compared to the parent strain (**Figure 2.8**).

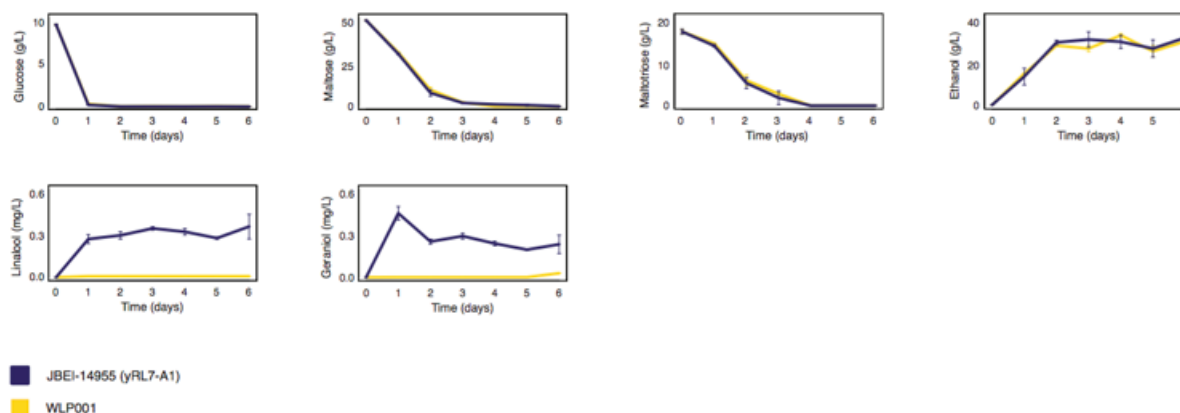


Figure 2.8. Fermentable sugars, ethanol, and monoterpene concentrations over the course of a 6-day fermentation. Engineered strain exhibits indistinguishable rates of sugar consumption and ethanol production. Error bars represent the standard deviation or 3 biological replicates.

Secondly, end-product accumulation can lead to cellular toxicity. Notably, previous studies have reported toxicity of monoterpenes at relatively low concentrations in *S. cerevisiae* due to cell wall stress^{80,81}. We therefore measured growth inhibition at various concentrations of linalool and geraniol (**Figure 2.9**). The concentrations of linalool and geraniol produced in the engineered strains were well below the minimum concentration of growth inhibition, suggesting that accumulation of these molecules at concentrations characteristic of finished beer does not adversely affect fermentation. We did not observe a significant correlation between remaining fermentable sugars and total monoterpene production (correlation of 0.28, p -value of 0.26), suggesting sugar consumption defects stemmed from pathway imbalance rather than metabolic burden.

Thirdly, metabolic pathway imbalance can lead to accumulation of toxic intermediates. These defects might be explained by imbalances in pathway enzyme expression that lead to increased accumulation of metabolite intermediates, as certain intermediates have been implicated as toxic at high cellular levels in various microorganisms⁸², including yeast⁸³.

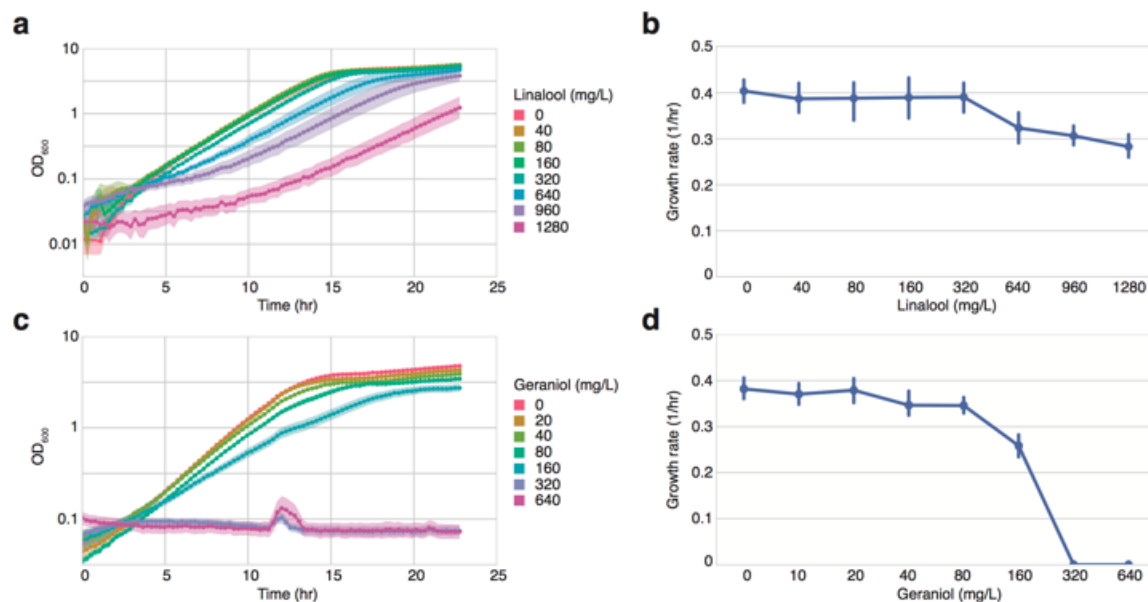


Figure 2.9. Toxicity of linalool and geraniol to brewer's yeast strain *WLP001*. **(a,c)** Growth of brewer's yeast in varying concentrations of linalool and geraniol. **(b,d)** Growth rates over a range of linalool and geraniol concentrations. Statistically significant growth defects are observed in media containing 640 mg/L linalool or 160 mg/L geraniol. Growth curves were calculated by averaging 6 biological replicates; shaded areas represent one standard deviation above and below the mean. Growth rates were calculated with a sliding window of 5 hours, solving for maximum growth rate. Growth rates are presented as the average of 6 biological replicates; error bars represent 95% confidence intervals.

To further explore the relationship between genetic design and monoterpene production, the relative abundance of the four modulated proteins was measured for each strain during the active phase of fermentation. Protein abundance was strongly correlated with previously characterized promoter strength (Supplementary Table 1 and **Figure 2.10**), demonstrating that the qualitative relationship between promoter strengths generally extends from a lab strain grown aerobically in rich medium to a brewing strain grown in industrial brewing conditions. Furthermore, total monoterpene production was correlated with tHMGR and FPPS* abundance and linalool production was correlated with t67-McLIS abundance, verifying that the selected genes indeed control monoterpene production as anticipated (**Figure 2.5e** and Supplementary Table 1). An interesting anomaly was that *ObGES* abundance was not correlated with geraniol production. We reasoned that since *ObGES* and t67-McLIS compete for GPP supply, variation in t67-McLIS abundance may obscure the relationship between *ObGES* and geraniol production. Indeed, we observed that the fraction of geraniol in total monoterpene composition correlated with the ratio between *ObGES* and t67-McLIS (p -value < 0.05; t -test). Together these findings were encouraging, as they firmly demonstrated that genetic design can be used to control monoterpene production and that the knowledge gained from our initial test set could guide subsequent design.

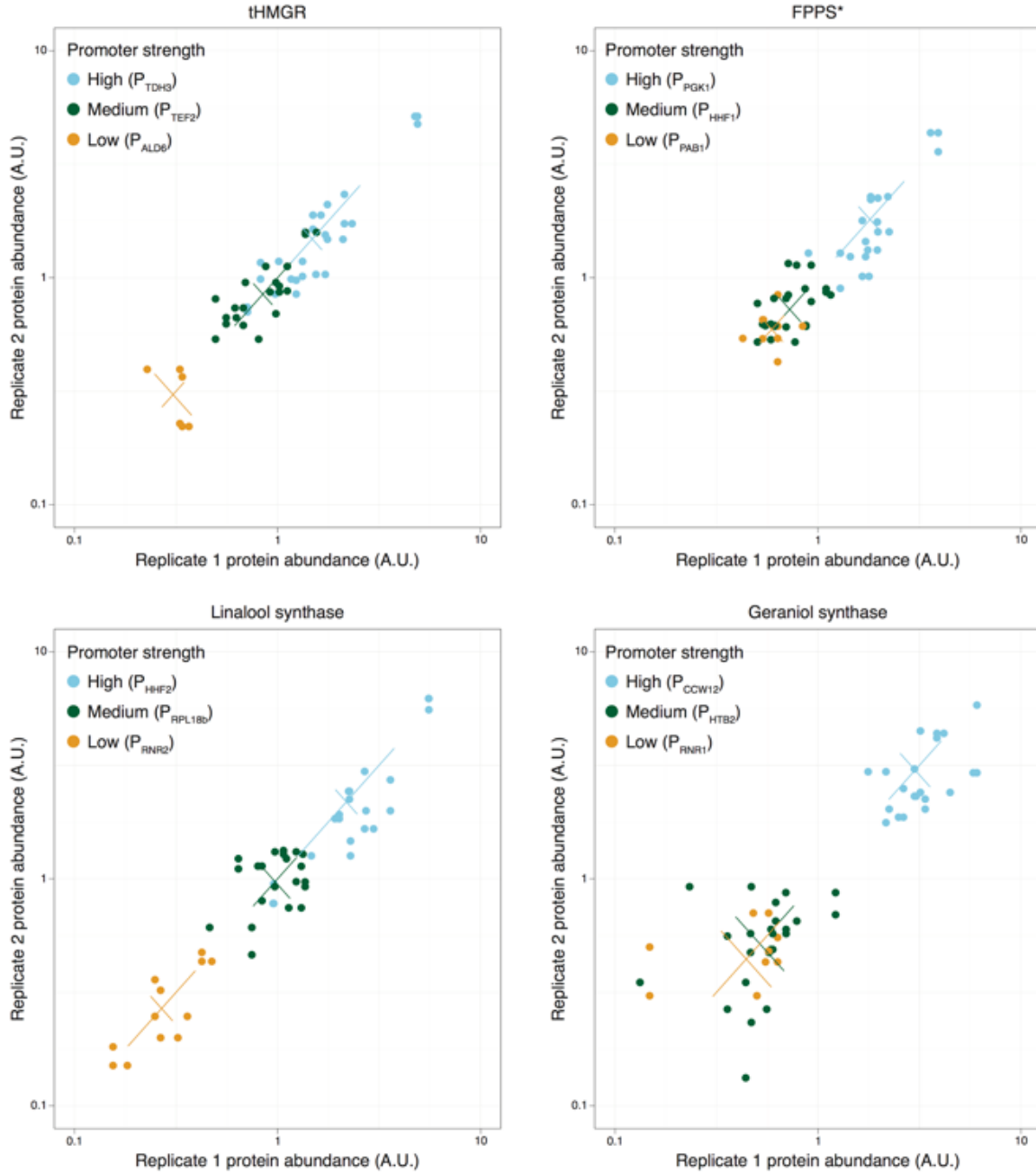


Figure 2.10. Sources of variation for protein abundance data from initial test set. Pairwise comparison of protein abundance values between experimental replicates illustrates magnitude of variation due to promoter, genetic context and experiment. Each point represents protein abundance values for a pair of biological replicates. Each pairwise comparison is color-coded by promoter. Variation due to promoter identity can be visualized as the distance between groups of colored dots along the line $y=x$. Variation due to genetic context is reflected by the deviation within a group of colored dots along the line $y=x$. Experimental variation is reflected by the deviation within a group of colored dots in the distance from the line $y=x$.

We next set out to generate a second iteration of designs with target production levels defined by three commercially hopped beers that span a wide range of

monoterpene concentrations and perceived hop flavor/aroma intensity. The salient trends observed in the test set informed two guiding principles: 1) to bias overall monoterpene production towards higher levels, designs were composed of strong promoters driving *tHMGR*, *FPPS**, and *ObGES* and 2) to ensure variation in the ratio of linalool to geraniol, designs encompassed a range of promoter strengths driving expression of *t67-McLIS* (Supplementary Table 9). We anticipated that applying these design principles towards desired performance characteristics would improve search efficiency. In order to evaluate the extent of improvement we established a mathematical modeling-based framework to predict the relationship between genetic design and monoterpene production (Methods, Supplementary Tables 2 and 3). Using this framework we predicted that the selected design principles significantly improved our search efficiency. Importantly, we observed a consistent improvement in comparing actual distance-from-target monoterpene levels between the first and second iteration strains (**Figure 2.11**).

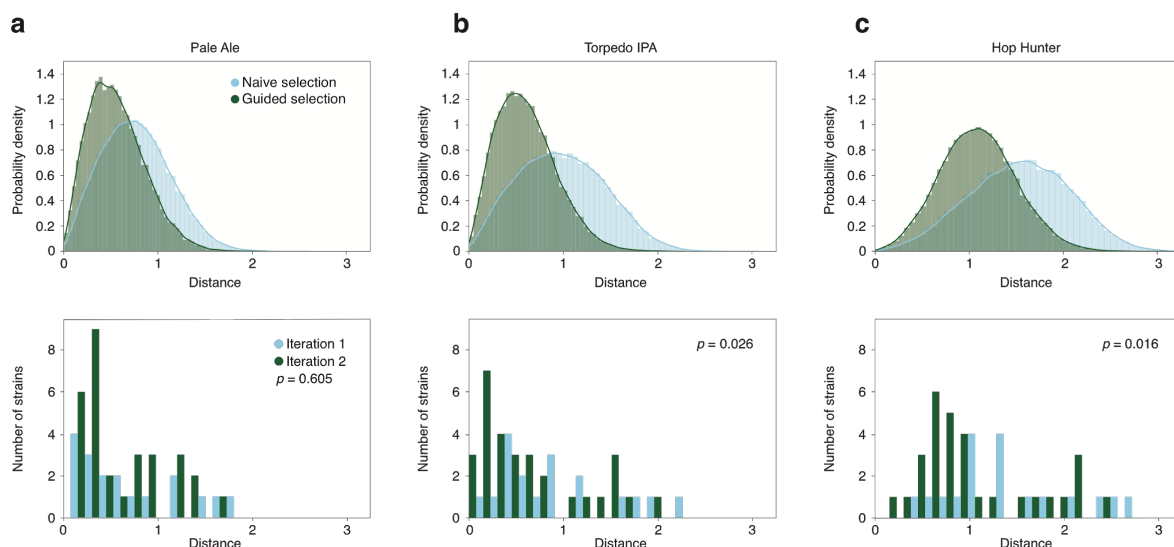


Figure 2.11. Evaluation of iterative genetic design refinement. Simulated (top panel) and measured (bottom panel) distance between engineered strain performance and target performance of commercial beers obtained from Sierra Nevada Brewing Company for first and second iteration strains. Target performance is defined based on Pale Ale (a), Torpedo IPA (b), and Hop Hunter IPA (c). In all cases, second iteration strains are closer to target performance than first iteration strains. p -values (t -test) reflect the degree of statistical significance with which second iteration strains are closer to target performance than first iteration strains.

The anticipated commercial value of generating hop flavor molecules through yeast biosynthesis is predicated on three assumptions: 1) because the conditions inside a fermenter can be precisely controlled, the final concentrations of yeast-biosynthesized monoterpenes in beer are likely to be more consistent compared with those given by conventional hop additions, 2) the biosynthesized monoterpenes linalool and geraniol confer hoppy flavor as perceived through human taste, and 3) the variation in hop flavor molecule concentrations correspond to differences on the order of those discernible by human taste. To test the consistency of yeast-biosynthesized monoterpene levels, replicate fermentations were performed at 8L scale with a subset of engineered strains. To test the consistency of hop-derived monoterpene levels, Cascade hop preparations

originating from five different farms in the Northwestern United States were used to supplement fermentations performed with the parent strain (**Figure 2.12a,b**). We observed little variation in final monoterpene concentrations between replicate samples fermented with engineered strains, whereas fermentations hopped with different preparations yielded significantly greater variation (linalool p -value $< 1 \times 10^{-5}$, geraniol p -value $< 1 \times 10^{-3}$; t -test) (**Figure 2.12a,b**).

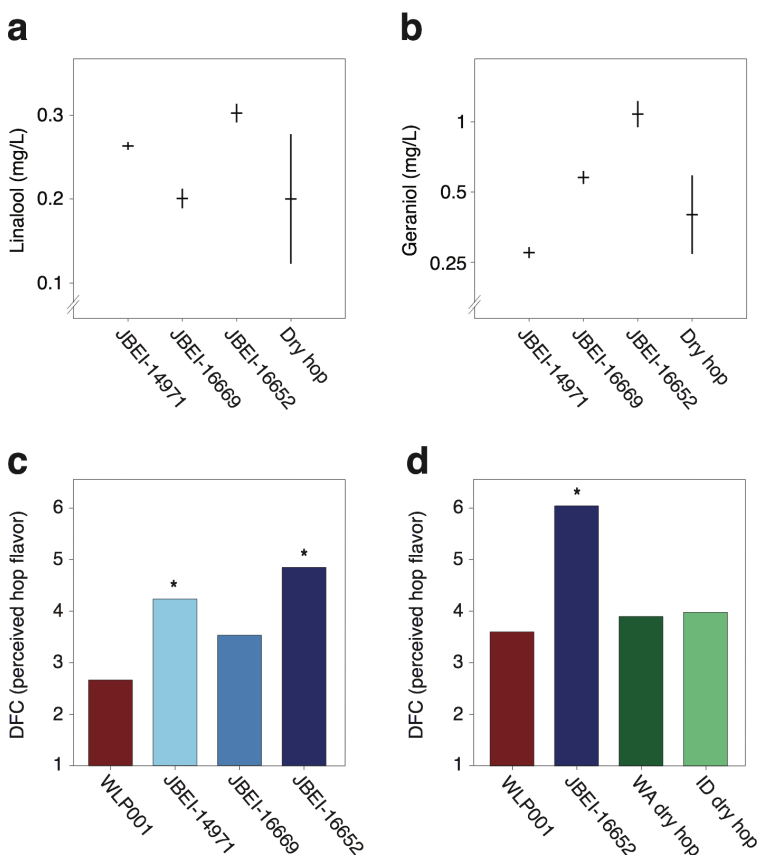


Figure 2.12. Characteristics of pilot-scale beer fermented with engineered strains. **a, b** Variation in linalool (**a**) and geraniol (**b**) concentrations of engineered brewing strain fermentations compared with variation in concentrations generated by traditional dry hopping. For engineered strain samples, horizontal lines correspond to the mean of three biological replicates. For traditional dry hopping, the horizontal line corresponds to the mean of five Cascade hop samples obtained from different farms. Vertical lines correspond to standard deviation. **c** Sensory analysis of the pilot-scale beers fermented with three engineered strains compared to beer fermented with the parental strain. **d** Sensory analysis of pilot-scale beers fermented with engineered strain JBEI- 16652 compared to beer fermented with the parental strain, with or without traditional Cascade dry-hopping. Asterisks (**c, d**) indicate statistically significant differences in hop aroma intensity as compared to the control beer (p -value < 0.05 ; Dunnett's test). Difference from control, DFC, was measured on a 9-point scale.

This result demonstrates that engineered strains biosynthesize monoterpenes more consistently than can be achieved by randomly selecting Cascade hop preparations from different farms. Next, to test whether yeast-biosynthesized monoterpenes conferred hop flavor, beer was produced in an authentic, pilot-scale brewhouse, following a recipe for a classic American Ale, using three engineered strains and the parent strain (*WLP001*) as a control (**Figure 2.13** and Supplementary Table

13). A panel of tasters determined that the finished beers exhibited a range of hop flavor/aroma intensity (**Figure 2.12c**). In addition, the apparent difference in hop flavor/aroma intensity between beer fermented with JBEI-16652 and JBEI-16669 was considerable, despite only a ~twofold difference in linalool and geraniol concentrations. Taken together, these results demonstrate that monoterpenes derived from yeast biosynthesis during fermentation give rise to hop flavor/aroma in finished beer and that biosynthesis provides greater consistency than traditional hopping. Finally, in order to compare the intensity of hop flavor conferred by traditional dry-hopping with the hop flavor conferred by engineered strains, fermentations were performed both with the parent strain with dry-hop additions as well as with JBEI-16652 without dry-hop additions (**Figure 2.13** and Supplementary Table 14). Conventionally dry-hopped beers consistently exhibited increased hop flavor as perceived by a sensory panel, however these effects were not statistically significant compared to the *WLP001* control (**Figure 2.12d**). In contrast, beer produced with JBEI-16652 again exhibited significantly higher hop flavor than the *WLP001* control. Similar monoterpene concentrations were observed between the two batches, demonstrating the consistent performance of the engineered strain.

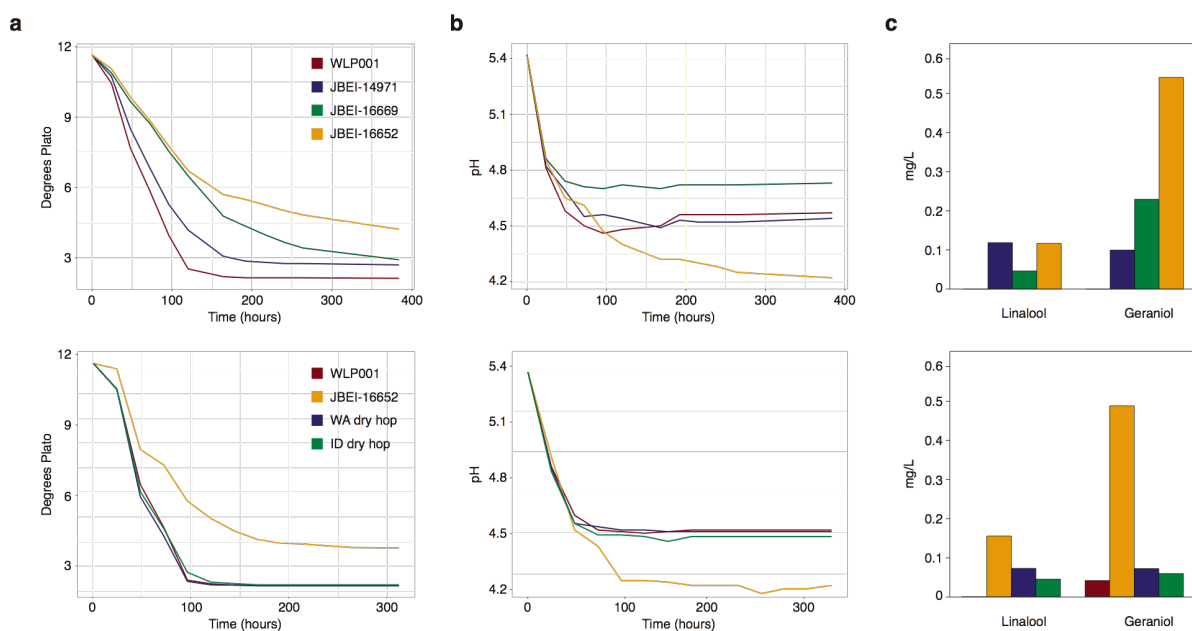


Figure 2.13. Sugar consumption, pH measurements, and GC/MS analysis of industrial-scale fermentations. Fermentable sugar consumption (**a**), pH measurements (**b**), and linalool and geraniol production (**c**) during industrial fermentations. The top row corresponds to the first set of pilot fermentations, the bottom row to the second set. Sugar consumption was measured using the Plato scale (for reference, a 12 °P wort contains 12 g sugar and other soluble components per 100 g wort). Interestingly, JBEI-14971 (in the first set) was determined to be significantly different from the *WLP001* control by sensory analysis yet it behaved most similar to the parent strain during fermentation.

2.5. Discussion

In this study, we have engineered brewer's yeast for production of flavor molecules ordinarily derived from hops. We developed new methods to overcome the

difficulties associated with stable integration of large constructs in industrial strains. Unlike classical microbial metabolic engineering efforts that focus on maximizing the titer of a single molecule, we focused on tuning the expression of key genes in a biosynthetic pathway to make precise concentrations of the desired flavors. This application promises to generate hop flavors with more consistency than traditional hop additions, as hop preparations are notoriously variable in the content of their essential oil and the flavor they impart to beer⁷¹. It should be noted that blending hop preparations from different sources can be used to reduce variation. However, blending is ultimately limited by practical constraints: In the best case, large craft breweries create one single hop blend per year, which fails to mitigate year-to-year variation. Our strategy is favored over plant or microbial bioprocess extraction because it avoids the use of non-renewable chemicals typical of industrial extraction. While historic consumer trepidation towards genetically engineered foods is of concern for widespread adoption, the general increase in consumer acceptance of such foods when tied to increased sustainability⁸⁴ is encouraging.

Previous studies have demonstrated the feasibility of engineering brewer's yeast by incorporation of heterologous genes^{85,86,87}; however, the scope and commercial relevance of these efforts have been limited, in part due to methodological difficulties of incorporating an array of large, genetically stable DNA constructs into industrial yeasts. Recent studies have resorted to alternative methods such as breeding hybrid strains⁸⁸. While this has proven to be a powerful approach for generating diverse aroma phenotypes, it is intrinsically limited to enzymes and aromas associated with native yeast metabolism. Here, we developed a complementary methodology that allows for stable incorporation of plant secondary metabolism genes into industrial brewer's yeast. We provide evidence that incorporating linalool and geraniol biosynthesis confers hop flavor to beer. We note that the full flavor imparted by traditional hopping is likely to rely on a more diverse bouquet of molecules. The methodologies described herein provide a foundation for generating more complex yeast-derived hop flavors, and broaden the possibilities of yeast-biosynthesized flavor molecules to those throughout the plant kingdom.

2.6. Methods

Cloning. All strains, expression plasmids, and additional plasmids used for strain construction are listed and described in Supplementary Tables 4-9. The sequence files corresponding to each plasmid can be found in the JBEI Public Registry (<https://public-registry.jbei.org/>)⁸⁹. Plasmids were propagated in *Escherichia coli* strain DH10B and purified by Miniprep (Qiagen, Germantown, MD). The "pathway" plasmids used to construct the engineered brewing strains were assembled by the standard Golden Gate method using type II restriction enzymes and T7 DNA Ligase (New England Biolabs, Ipswich, MA)^{72,90} (for additional detail, see schematized assembly strategy in Supplementary Fig. 3). All other plasmids generated in this study were constructed by Gibson assembly⁹¹ using Gibson assembly master mix (New England Biolabs, Ipswich, MA). Constructs were designed using Device Editor bioCAD software⁹², and assembly primers were generated with j5 DNA assembly design automation software⁹³ using the default settings. PCR amplification was performed using PrimeSTAR GXL DNA

polymerase according to the manufacturer's instructions (Takara Bio, Mountain View, CA). Genes coding for full-length linalool and geraniol synthases were ordered either from IDT (San Diego, CA) as G-blocks or from Life Technologies (Carlsbad, CA) as DNA strings. The coding sequences of heterologous genes in all plasmids were validated by Sanger sequencing (Genewiz, South Plainfield, NJ and Quintara, South San Francisco, CA).

Strain construction. Yeast lab strains were transformed by the high efficiency lithium acetate method⁹⁴. Strains were cultivated in Yeast extract + Peptone + Dextrose (YPD) medium unless otherwise noted. To select for transformants containing auxotrophic complementation cassettes, transformed cells were plated on standard dropout medium (Sunrise Science Products, San Diego, CA). To select for transformants containing drug resistance cassettes, cells were recovered in YPD medium for 4 h after transformation, and then plated on YPD medium supplemented with 200 µg/L Geneticin (Sigma-Aldrich, St. Louis, MO) or Hygromycin B (Sigma-Aldrich, St. Louis, MO). Minor modifications were made to cultivation conditions for brewer's yeast transformations: pre-transformation cultures were grown in YPD medium supplemented with 200 mg/L adenine sulfate at 20°C in glass test tubes with shaking at 200 rpm. A single colony was used to inoculate an initial 5 mL culture, which was grown overnight to turbidity. This culture was used to inoculate a second 5 mL culture to an OD₆₀₀ of 0.01, which was grown for 18 h. The second culture was then used to inoculate 50 mL cultures in 250 mL Erlenmeyer flasks to OD₆₀₀ of 0.05. After ~8 hours of growth, strains were transformed by the lithium acetate method⁹⁴, cells were recovered in YPD medium for 4 h, plated on YPD supplemented with 200 µg/L Geneticin, and then grown for 5-7 d at 20°C.

DNA used for genomic integration was prepared either by PCR-amplifying plasmid DNA or by digesting a plasmid with restriction enzymes. For construction of the GPP-hyper-producing strain, integration fragments were amplified from the corresponding plasmids by PCR (Supplementary Table 5). For construction of pathway-integrated brewing strains, plasmid DNA was linearized by restriction digestion with NotI-HF and PstI-HF (New England Biolabs, Ipswich, MA) (Supplementary Tables 8 and 9).

All integration events were confirmed by diagnostic PCR using GoTaq Green Master Mix (Promega, Madison, WI). For brewer's yeast strains, homozygosity at the integration locus was tested using primers targeted to the 5' and 3' junctions of desired allele and the parental allele. The identity of the multi-gene integration was verified with primers targeted to each of the four promoter/gene junctions.

Screening synthases. For the linalool and geraniol synthase screening, single colonies were picked from the transformation plate and used to inoculate cultures in 5 mL CSM -Leu (Sunrise) + 2% raffinose (Sigma-Aldrich, St. Louis, MO) medium. After 24 h, the pre-cultures were diluted into fresh CSM -Leu + 2% galactose (Sigma-Aldrich, St. Louis, MO) medium to an OD of 0.05 and grown for 72 h with shaking at 200 rpm. An organic overlay was added 24 h after inoculation to capture hydrophobic monoterpenes. Decane was used as the overlay for the cultures expressing linalool synthase and dodecane was used for those expressing geraniol synthase. The overlay was chosen so

as to minimize overlap of retention times between solvent and product for subsequent GC/MS analysis.

Microaerobic fermentation. Strains were streaked on YPD medium and grown for 2 days at 25°C. Single colonies were used to inoculate initial 2 mL precultures in 24-well plates (Agilent Technologies, Santa Clara, CA), which were grown for 3 days at 20°C with shaking at 200 rpm. Strains were grown in a base medium composed of 100 g/L malt extract (ME) (Sigma-Aldrich, St. Louis, MO). Each well contained a 5 mm glass bead (Chemglass Life Sciences, Vineland, NJ). The resulting cultures were used to inoculate second 6 mL precultures in fresh 24-well plates to an OD of 0.1, which were then grown for 3 days at 20°C with shaking at 120 rpm. The resulting cultures were then used to inoculate 25 mL cultures in glass test tubes to an OD of 1.0. These cultures were equipped with a one-way airlock for microaerobic fermentation and grown for 5 days at 20°C. Test tubes were vortexed for 30 s every 24 h.

High-performance liquid chromatography. Maltotriose, maltose, glucose, and ethanol were separated by High Performance Liquid Chromatography (HPLC) and detected by a refractive index (RI) detector. On day 5, fermentation samples were centrifuged at 18,000g for 5 min, filtered using Costar® Spin-X® Centrifuge Tube Filters, 0.22-µm pore, transferred to HPLC tubes, and loaded into an Agilent 1100 HPLC equipped with an Agilent 1200 series auto-sampler, an Aminex HPX-87H ion exchange column (Bio-Rad, Hercules, CA), and an Agilent 1200 series RI detector. Metabolites were separated using 4 mM H₂SO₄ aqueous solution with a flow rate of 0.6 mL/min at 50°C. Absolute sample concentrations were calculated using a linear model generated from a standard curve composed of authentic maltotriose, maltose, glucose, and ethanol standards (Sigma-Aldrich, St. Louis, MO) diluted in water over a range of 0.2 - 20 g/L. All data are provided in Supplementary Table 11.

Monoterpene quantification. Monoterpenes were quantified by gas chromatography–mass spectrometry (GC/MS) analysis, using an Agilent GC system 6890 series GC/MS with Agilent mass selective detector 5973 network. In all experiments, 1 µL of sample was injected (splitless), using He as the carrier gas onto a CycloSil-B column (Agilent, 30-m length, 0.25-mm inner diameter (i.d.), 0.25-µm film thickness, cat. no. 112-6632). The carrier gas was held at a constant flow rate of 1.0 mL/min and EMV mode was set to a gain factor of 1.

Sampling, oven temperature schedule, and ion monitoring was optimized for each experiment: for quantifying linalool and geraniol production in terpene synthase screens, the samples were spun down and the organic phase (solvent overlay) was collected, diluted 1:10 in ethyl acetate (Sigma-Aldrich, St. Louis, MO), transferred to a glass GC vial and injected into the GC column. For samples corresponding to the linalool synthase screen, the oven temperature was held at 50°C for 12 min followed by a ramp of 10°C/min to a temperature of 190°C and a ramp of 50°C/min to a final temperature of 250°C, and then held at 250°C for 1 min. The solvent delay was set to 20 min, and the MS was set to SIM mode for acquisition, monitoring *m/z* ions 80, 93, and 121. For samples corresponding to the geraniol synthase screen, the oven temperature was held at 50°C for 5 min, then ramped at 30°C/min to a temperature of 135°C, then ramped at 5°C/min to a temperature of 145°C, then ramped at 30°C/min to

a temperature of 250°C, and held at 250°C for 1 min. The solvent delay was set to 10.8 min and the MS was set to monitor m/z ions 69, 93, 111, and 123. For quantifying linalool and geraniol in micro-aerobic fermentations performed with brewer's yeasts, samples were extracted on day 5 using ethyl acetate. Fermentation samples were collected and spun down, 1600 μ L of the supernatant was mixed with ethyl acetate at a 4:1 ratio in a 96-well plate, the plate was sealed and vortexed for 2 min, then spun at 3000g for 5 min, and 30 μ L of the ethyl acetate was transferred into a glass GC vial. The resulting preparation was injected into the GC column. For quantifying linalool and geraniol in various commercial beers, 2 mL of ethyl acetate was added to 8 mL of the beer in glass tubes (Kimble Chase, Rockwood, TN). This was mixed by hand for 2 min and spun at 1000g for 10 min. 30 μ L of the ethyl acetate layer was transferred to glass GC vials, and the resulting preparation was injected into the GC column. For both the micro-aerobic fermentation experiments and sampling of commercial beers, the oven temperature was held at 50°C for 5 min followed by a ramp of 5°C/min to a temperature of 200°C and a ramp of 50°C/min to a final temperature of 250°C, and then held at 250°C for 1 min. The solvent delay was set to 5 min and the MS was set to monitor m/z ions 55, 69, 71, 80, 81, 93, 95, 107, 121, 123, and 136.

Peak areas for linalool and geraniol were quantified using MSD Productivity ChemStation software (Agilent Technologies, Santa Clara, CA). Absolute sample concentrations were calculated using a linear model generated from a standard curve composed of authentic linalool and geraniol standards (Sigma-Aldrich, St. Louis, MO). For monoterpene synthase screening experiments, standards were diluted in ethyl acetate over a range of 0.2 - 50 mg/L. For the micro-aerobic fermentation experiments and sampling of commercial beers, standards were spiked into a preparation extracted from the parent strain fermentation sample (i.e. a control preparation used to ensure accurate baseline signal) over a range of 0.2 - 10 mg/L. In calculating actual concentrations, apparent concentrations were scaled based on dilution or concentration in GC injection preparation.

Proteomics. Protein abundance data are reported in Supplementary Table 12. Culture (5 mL) was sampled after 2 d, vortexed, and spun at 3000g for 5 min. The supernatant was discarded, and the pellet was flash frozen. Plate-based cell pellets were lysed by chloroform-methanol precipitation, described below, while samples in tubes were lysed by re-suspending the pellets in 600 μ L of yeast lysis buffer (6 M urea in 500 mM ammonium bicarbonate) followed by bead beating with 500 μ L Zirconia/Silica beads (0.5 mm diameter; BioSpec Products, Bartlesville, OK). Samples in tubes were bead beat for five cycles of 1 min with 30 s on ice in between each cycle. Subsequently, they were spun down in a benchtop centrifuge at maximum speed for 2 min to pellet cell debris, and the clear lysate was transferred into fresh tubes. Plate-based cell lysis and protein precipitation was achieved by using a chloroform-methanol extraction⁹⁵. The pellets were re-suspended in 60 μ L methanol and 100 μ L chloroform, then 50 μ L Zirconia/Silica beads (0.5 mm diameter; BioSpec Products, Bartlesville, OK) were added to each well. The plate was bead beat for five cycles of 1 min with 30 s on ice in between each cycle. The supernatants were transferred into a new plate and 30 μ L water was added to each well. The plate was centrifuged for 10 min at maximum speed to induce the phase separation. The methanol and water layers were removed, then 60

μL of methanol was added to each well. The plate was centrifuged for another 10 min at maximum speed then the chloroform and methanol layers were removed and the protein pellets were dried at room temperature for 30 min prior to re-suspension in 100 mM ammonium bicarbonate with 20% methanol.

The protein concentration of the samples was measured using the DC Protein Assay Kit (Bio-Rad, Hercules, CA) with bovine serum albumin used as a standard. A total of 50 μg protein from each sample was digested with trypsin for targeted proteomic analysis. Protein samples were reduced by adding tris 2-(carboxyethyl)phosphine (TCEP) to a final concentration of 5 mM, followed by incubation at room temperature for 30 min. Iodoacetamide was added to a final concentration of 10 mM to alkylate the protein samples then incubated for 30 min in the dark at room temperature. Trypsin was added at a ratio of 1:50 trypsin:total protein, and the samples were incubated overnight at 37°C.

Peptides were analyzed using an Agilent 1290 liquid chromatography system coupled to an Agilent 6460 QQQ mass spectrometer (Agilent Technologies, Santa Clara, CA). The peptide samples (10-20 μg[LC2]) were separated on an Ascentis Express Peptide ES-C18 column (2.7 μm particle size, 160 Å pore size, 5 cm length x 2.1 mm i.d., coupled to a 5 mm x 2.1 mm i.d. guard column with similar particle and pore size; Sigma-Aldrich, St. Louis, MO), with the system operating at a flow rate of 0.400 mL/min and column compartment at 60°C. Peptides were eluted into the mass spectrometer via a gradient with initial starting condition of 95% Buffer A (0.1% formic acid) and 5% Buffer B (99.9% acetonitrile, 0.1% formic acid). Buffer B was held at 5% for 1.5 min, then increased to 35% B over 3.5 min. Buffer B was further increased to 80% of 0.5 min where it was held for 1 min, then ramped back down to 5% B over 0.3 min where it was held for 0.2 min to re-equilibrate the column to the initial starting condition. The peptides were ionized by an Agilent Jet Stream ESI source operating in positive-ion mode with the following source parameters: Gas Temperature = 250°C, Gas Flow = 13 L/min, Nebulizer Pressure = 35 psi, Sheath Gas Temperature = 250°C, Sheath Gas Flow = 11 L/min, VCap = 3,500 V. The data were acquired using Agilent MassHunter, version B.08.00. Resultant data files were processed by using Skyline⁹⁶ version 3.6 (MacCoss Lab, University of Washington, Seattle, WA) and peak quantification was refined with mProphet⁹⁷ in Skyline.

Data analysis. Data analysis was performed using the R statistical programming language⁹⁸. Additional libraries were used for data visualization functionalities^{99–103}. For protein and metabolite analysis heatmaps (**Figure 2.5e**), relative levels were reported as follows: promoter strengths were represented as a fraction of their previously reported rank order⁷² ranging from P_{RNR2} (0) to P_{TDH3} (1). Feature scaling was used to standardize the range of protein, monoterpene, and sugar abundances. Let s_i equal the $\log_{10}$ -transformed abundance value for species S in strain i . Normalized values were computed according to equation (1) as:

$$s_i' = \frac{s_i - \min(S)}{\max(S) - \min(S)}$$

For sugar analysis, unfermented malt extract was included in max/min calculations. For fermentable sugars (i.e. maltotriose, maltose, glucose) the scaled values were subtracted from 1 in order to represent proximity to desired sugar consumption profile.

The distance metric of an engineered strain with respect to a given commercial beer was calculated using the Manhattan length as the distance of monoterpene production from beer monoterpene concentrations and the distance in sugar consumption from the parent strain. First, the difference between $\log_{10}$ -transformed values of engineered strain monoterpene concentration and target beer monoterpene concentration was calculated for each species, linalool and geraniol. Second, the absolute values of these differences were calculated. Finally, the resulting values, together with the fraction of total sugar remaining after fermentation, were averaged.

Mathematical modeling. Three different models were constructed in Python to predict monoterpene production from protein levels (for detailed description and implementation, see Appendix 5.1). Both the Gaussian regressor and linear models were implemented using Scikit Learn¹⁰⁴. Additional equations needed to describe the linear model are given in Supplementary Table 3. Equations describing the Michaelis-Menten kinetics model are given in Appendix 5.1. Kinetic parameters were scraped from the literature. Free parameters were included to convert relative protein counts to absolute protein values. Additionally, a parameter β determined the relative ratio between the endogenous FPPS and FPPS*.

Both the linear and Gaussian regressor models were fit using standard methods from the Scikit Learn library. The kinetic model was manually constructed without external libraries. To fit the kinetic model, a differential evolution algorithm was used to perform parameter optimization on a nonlinear cost function. Specifically, the sum of the squared residual error of the model predictions from the first iteration strains was minimized with respect to the previously described parameters. The kinetic coefficients were bounded to vary over an order of magnitude from the values described in the literature. In order to cross validate the models and minimize overfitting, a leave-one-out methodology was applied to each model. The error residuals from this cross-validation technique are reported in Supplementary Table 2.

Analysis performed for predicting the extent of performance improvement for second iteration strains (**Figure 2.11**) compared with randomly designed strains is described in Appendix 5.1.

Toxicity assay. OD₆₀₀ measurements were taken in 48-well clear flat bottom plates (Corning Inc., Corning, NY) using a Tecan Infinite F200 PRO reader, with acquisition every 15 min. Analysis was performed using custom python scripts. Growth curves were calculated by averaging 6 biological replicates; shaded areas represent one standard deviation from the mean. Growth rates were calculated with a sliding window of 5 hours, solving for maximum growth rate. Growth rates are presented as the average of 6 biological replicates; error bars represent 95% confidence intervals (**Figure 2.9**).

Pilot fermentations. Strains were streaked on YPD medium and grown for 2 days at 25°C. Single colonies were used to inoculate initial 5 mL cultures in glass test tubes, which were grown for 2 days at 20°C with shaking at 200 rpm. The resulting cultures were used to inoculate 1 L cultures in 2 L glass Erlenmeyer flasks, which were then grown for 3 days at 20°C with shaking at 200 rpm. Strains were grown in a base medium composed of 100 g/L malt extract (ME) (Sigma-Aldrich, St. Louis, MO). The resulting cultures were then used to inoculate industrial fermentations in wort produced in a 1.76 hL pilot brewery.

For the first set of fermentations, 35 kg of 2-Row malt was milled and added to 105 L of DI water treated with 79.15 g of brewing salts. Mashing was performed for 30 min at 65°C, 10 min at 67°C, and 10 min at 76 °C. The wort was allowed to recirculate for 10 min and was separated by lautering. Sparging occurred for 58 min, giving a final pre-boil volume in the brew kettle of 215 L. The wort was boiled until it reached a final volume of 197 L and a gravity of 11.65 °Plato. Kettle additions included 125 g of Magnum hop pellets, 15.1 g of Yeastex yeast nutrients, and 15 g Protofloc (Murphy and Son, Nottingham, United Kingdom). Ingredients were sourced from Brewers Supply Group (Shakopee, MN) except where otherwise noted. After the wort was separated from the hot trub, it was transferred to four 56 L custom fermenters (JVNW, Canby, OR), each filled to 40 L. The beers were fermented at 19°C until they reached terminal gravity, held for an additional 24 hours for vicinal diketone (VDK) removal, and then cold conditioned at 0°C. The length of fermentation, and in turn the length of cold conditioning, was strain dependent. Samples were taken every 24 hours to measure °Plato and pH (**Figure 2.13**). The resulting beer was filtered under pressure and carbonated prior to storage in 7.75 gallon kegs. Samples were collected during the kegging process for Alcolyzer (Anton Paar, Ashland, VA) analysis (Supplementary Table 13).

For the second set of fermentations, 35 kg of 2-Row malt was milled and added to 105 L of DI water treated with 79 g of brewing salts. Mashing was performed for 30 min at 65°C, 10 min at 67°C, and 10 min at 76 °C. The wort was allowed to recirculate for 10 min and was separated by lautering. Sparging occurred for 52 min, giving a final pre-boil volume in the brew kettle of 214 L. The wort was boiled until it reached a final volume of 194 L and a gravity of 11.25 °Plato. Kettle additions included 97.01 g of Galena hop pellets, 15.1 g of Yeastex yeast nutrients, and 15 g Protofloc (Murphy and Son, Nottingham, United Kingdom). Ingredients were sourced from Brewers Supply Group (Shakopee, MN) except where otherwise noted. After the wort was separated from the hot trub, it was transferred to four 56 L custom fermenters (JVNW, Canby, OR), each filled to 40 L. The beers were fermented at 19°C until they reached terminal gravity, held for an additional 24 hours for vicinal diketone (VDK) removal, and then cold conditioned at 0°C. The length of fermentation, and in turn the length of cold conditioning, was strain dependent. Samples were taken every 24 hours to measure °Plato and pH (**Figure 2.13**). After 48 hours at 0°C, 88.5 g Cascade dry hops (either from Washington or from Idaho) were added to two fermenters containing parent strain *WLP001*. The dry hops were left on the beer at 1.67°C for 1 week before filtering. The resulting beer was filtered under pressure and carbonated prior to storage

in 7.75 gallon kegs. Samples were collected during the kegging process for AlcoLyzer (Anton Paar, Ashland, VA) analysis (see Supplementary Table 14).

Sensory analysis. Institutional Review Board approval for human research was obtained from the UC Berkeley Office for Protection of Human Subjects (CPHS protocol number 2017-05-9941). The Committee for Protection of Human Subjects reviewed and approved the application under Category 7 of federal regulations.

Panelists: sensory analysis of the brewed beer was conducted at Lagunitas Brewing Company (Petaluma, CA). The first panel consisted of 27 employee participants (17 males and 10 females), the second of 13 employee participants (11 males and 2 females), ranging in experience from 2 to 154 tasting sessions attended in calendar year 2017. Ages ranged from mid-20s to 50s. All participants received basic sensory training per Lagunitas standards.

Sensory Analysis: samples of 2 ounces were presented in clear 6 oz brandy glasses (Libbey, Toledo, OH). Each panelist received 5 glasses, one control and four samples (one blind control and 3 variables) arranged randomly by balanced block design. Block design and data gathering were accomplished using EyeQuestion® software (Logic8 BV, Netherlands). In a single sitting, panelists were asked to rank hop aroma intensity as compared to the control on a 9-point ordinal scale anchored on one end with “No difference” and the other end with “Extreme difference”.

Data Analysis: data were analyzed using Dunnett’s test in conjunction with one-way ANOVA using EyeOpenR® (Logic8 BV, Netherlands). Analysis was performed at the 95% confidence level. The blind control is used as the reference sample to account for any scoring bias that might occur.

Dry hopping for evaluation of variation between hop preparations. Parental strain *WLP001* was streaked on YPD medium and grown for 2 days at 25°C. A single colony was used to inoculate an initial 50 mL preculture in a 250 mL glass Erlenmeyer flask, which was grown for 1 day at 20°C with shaking at 200 rpm. The strain was grown in a base medium composed of 100 g/L malt extract (ME) (Sigma-Aldrich, St. Louis, MO) supplemented with YPD. The resulting culture was used to inoculate a 1 L preculture in a 2L glass Erlenmeyer flask, which was then grown for 2 days at 20°C with shaking at 200 rpm. The resulting culture was then used to inoculate four 2 L cultures in 4 L glass Erlenmeyer flasks, which were then grown for 1 day at 20°C with shaking at 200 rpm. The resulting cultures were then used to inoculate 8 L cultures in 3-gallon glass carboys (Midwest Supplies, Roseville, MN). These cultures were equipped with a one-way airlock for microaerobic fermentation and grown for 6 days at 20°C. In the meantime, 5 different Cascade hop samples grown on farms across the Pacific Northwest were obtained from YCH Hops (Yakima, WA). The hop samples were ground using a mortar and pestle and liquid nitrogen. On day 6, samples were taken from the fermentations (as un-hopped controls) and 25 grams of hops was added to each fermentation. Hops were left to steep for 3 days after which samples were collected for GC/MS analysis.

Batch-to-batch variation. Strains were streaked on YPD medium and grown for 2 days at 25°C. Single colonies were used to inoculate initial 5 mL precultures in glass test tubes, which were grown for 2 days at 20°C with shaking at 200 rpm. Strains were grown in a base medium composed of 100 g/L malt extract (ME) (Sigma-Aldrich, St. Louis, MO). The resulting cultures were used to inoculate 500 mL precultures in 2L glass Erlenmeyer flasks, which were then grown for 1 day at 20°C with shaking at 200 rpm. The resulting cultures were then used to inoculate 8 L cultures in 3-gallon glass carboys (Midwest Supplies, Roseville, MN). These cultures were equipped with a one-way airlock for microaerobic fermentation and grown for 12 days at 20°C. Samples were taken on day 12 for GC/MS analysis.

Data availability. The authors declare that all data supporting the findings of this study are available within the paper and its supplementary information files. Sequence data and strains generated in this study have been deposited in the JBEI public registry. See Supplementary Tables 4-9 for construct sequences and strain information.

3. Chapter 3 - Additional modifications to linalool and geraniol producing strains and possibilities for the reconstitution of the full hop aroma

3.1. Further engineering work: citronellol

Beer fermented with strains engineered to express the linalool and geraniol synthases were described in sensory analyses as having a desirable orange-citrus character. Neither linalool nor geraniol is generally described to have this character; however, the native yeast NADPH oxidoreductase encoded by *OYE2* is known to convert geraniol to citronellol during fermentation^{14,105}, and citronellol is known to confer this particular organoleptic property¹⁰⁶. With the improved sensitivity of a triple quadrupole mass spectrometer, compared to the single quadrupole MS used in previous work, citronellol was detected in samples of beer fermented with the initial 48 engineered strains at concentrations well above the reported flavor detection threshold¹⁰⁷. Strain JBEI-16652, which was used in side-by-side fermentations with the parent strain and was significantly hoppier even than the parent strain with dry hop additions, was found to produce more citronellol than geraniol (0.4 mg/L geraniol, 0.6 mg/L citronellol).

Based on the desirability of the citronellol aroma in beer and the existence of citronellol in engineered strains, we set out to test whether modulation of *OYE2* could affect the concentration of citronellol in finished beer. We generated two strains that differ only in promoter - weak or strong - driving expression of *OYE2*. In addition to *OYE2*, strains contained *tHMG1*, *ERG20**, and *ObGES* (but no linalool synthase). Strains were grown at small scale (25 mL) under microaerobic fermentation conditions in culture vials equipped with a one-way airlock to mimic industrial brewing conditions. Samples were taken on day 5 of fermentation, and terpenes were quantified by solid-phase microextraction (SPME) on a Varian triple quadrupole GC-MS/MS. Growth and fermentation capacity was also characterized for each strain. We found that driving *OYE2* expression from a stronger promoter resulted in an ~8-fold difference in total citronellol concentration after 5 days of test-tube-scale microaerobic fermentation (**Figure 3.2**). These results suggest that citronellol production can be affected by modulating the gene regulatory program of geraniol biosynthesis genes in conjunction with *OYE2*.

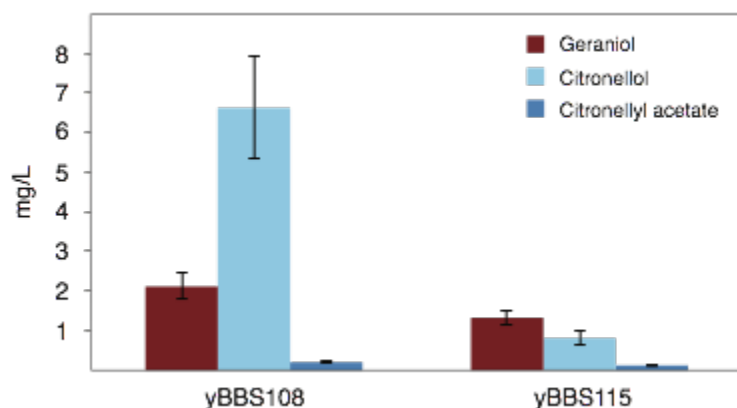


Figure 3.1. Quantification of monoterpenes produced by two *OYE2* overexpressing strains. *OYE2* is expressed from a higher strength promoter in strain yBBS108, resulting in increased citronellol production. Citronellyl acetate, a derivative of citronellol, was also quantified for each strain. Error bars correspond to mean $\pm$ standard deviation of three biological replicates.

3.2. Further engineering work: truncation of geraniol synthase

In plants, monoterpenes are biosynthesized in chloroplasts; plant monoterpene synthases, therefore, typically contain an N-terminal plastid targeting sequence (PTS) composed of 20-80 generally polar amino acids, which is cleaved to produce a mature protein. While full-length proteins can show activity in yeast, truncation of the PTS sequence often improves expression and activity^{108,109}. In preliminary experiments, a screen of geraniol synthase variants revealed that heterologous expression of the full-length version from basil led to geraniol production at concentrations typical of those found in a pale ale. Therefore, the original 48 strains contain a full-length geraniol synthase. Measurements of the relative abundance of the four modulated proteins in each strain revealed that total monoterpene production was correlated with abundance of upstream mevalonate pathway genes, and linalool production was correlated with linalool synthase abundance. However, geraniol production was not correlated with geraniol synthase abundance. Our initial explanation for this discrepancy was that variation in linalool synthase obscured the relationship between geraniol synthase and geraniol, as both synthases compete for GPP precursor supply. However, a second explanation is that protein instability caused by the presence of the PTS leads to rapid degradation of geraniol synthase over the course of fermentation (and thus little expression by the end of fermentation, despite its activity at the beginning). Proteomic measurements at the beginning of fermentation, correlating with the peak in geraniol production, may have shown better correlation between geraniol production and geraniol synthase. As an alternative, we set out to build strains containing truncated geraniol synthase (tGES), hopefully resulting in more stable geraniol synthase expression and more predictable geraniol production.

Two new strains were generated by making modifications at the promoter-geraniol synthase combination relative to JBEI-16652. Since preliminary experiments had indicated that the truncated synthase leads to excess geraniol production (**Figure**

3.2), and since these strains exhibit high expression of upstream mevalonate pathway genes, we used glycolytic promoters predicted to have low expression to drive tGES. Two additional strains were generated that exhibit lower expression of upstream mevalonate pathway genes relative to strain JBEI-16652, modifications also expected to reduce geraniol production. Strains were grown at small scale (25 mL) under microaerobic fermentation conditions in culture vials equipped with a one-way airlock to mimic industrial brewing conditions. Samples were taken on day 5 of fermentation and terpenes were quantified by solid-phase microextraction (SPME) on a Varian triple quadrupole GC-MS/MS. Growth and fermentation capacity was also characterized for each strain.

Low-strength promoters driving expression of tGES resulted in similar or higher geraniol concentrations compared to strain JBEI-16652 (which has the high-strength *HHF2* promoter driving the full-length synthase, see **Figure 3.2a**). However, promoters did not always behave as expected, likely a limitation associated with the use of glycolytic promoters in fermentation conditions. For example, strains yBBS116 and yBBS117 differ only by the identity of the tGES promoter, but yBBS117 produces 3.5-fold more geraniol with a much lower-strength promoter (**Figure 3.2b**). Predicting geraniol production from our current information is therefore not possible. Proteomic or transcriptomic data might reveal that a supposed “weak” promoter is in fact strongly induced in fermentation conditions, but monoterpene levels are currently at odds with what we would expect based on promoter rank. At the very least, however, these strains have expanded the range of possible geraniol production. And as an unexpected effect, these changes at a single promoter-gene combination affected the production of all terpenes, leading to higher linalool concentrations as well.

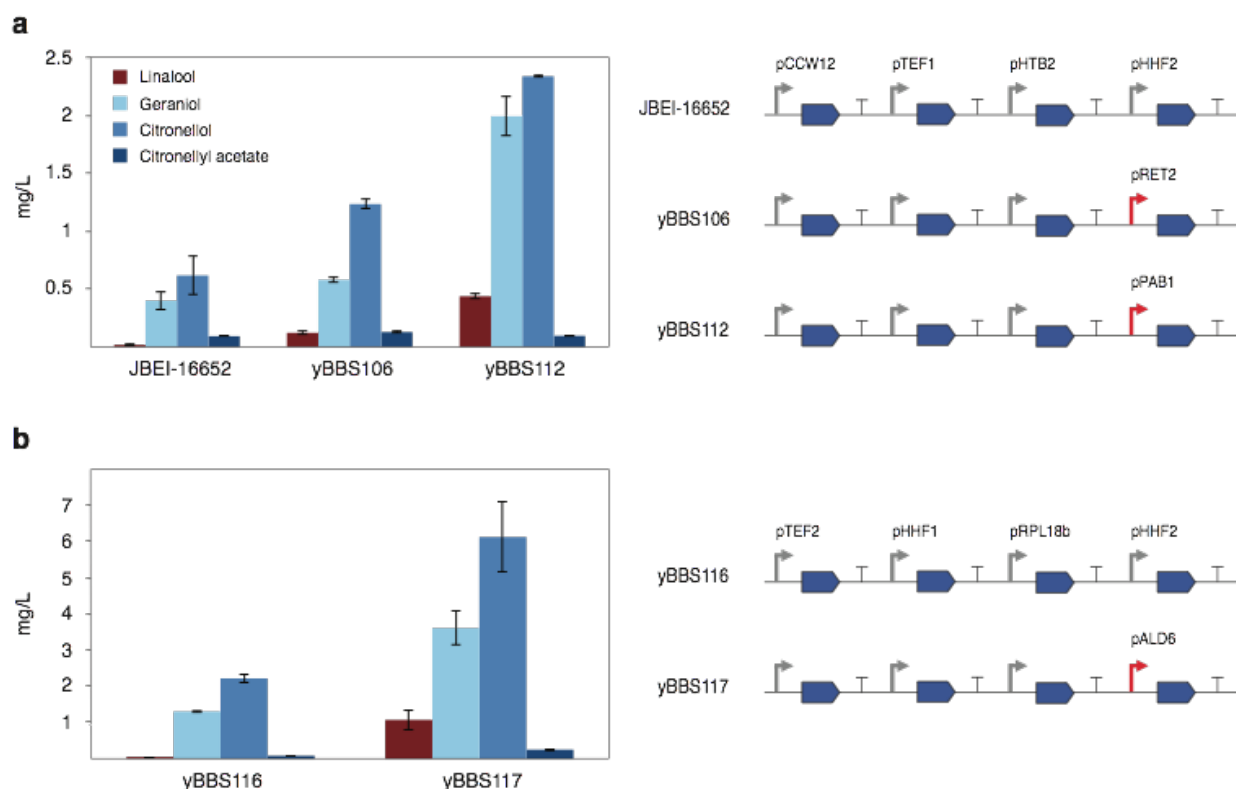


Figure 3.2. Quantification of monoterpenes produced by strains containing truncated geraniol synthase. **(a)** Second iteration strain JBEI-16652 (with full-length geraniol synthase) and newly designed strains containing modifications at the promoter-geraniol synthase combination relative to JBEI-16652. Genetic designs are depicted on the right (genes, from right to left, are *tHMG1*, *ERG20**, *t67-McLIS*, and *ObGES*); promoter modifications are highlighted in red. **(b)** Strains with lower mevalonate pathway expression relative to JBEI-16652. Genetic designs are depicted on the right (genes, from right to left, are *tHMG1*, *ERG20**, *t67-McLIS*, and *ObGES*). Error bars correspond to mean $\pm$ standard deviation of three biological replicates.

3.3. Future work

Hop essential oil, defined as containing components that are volatile in steam and often isolated by distillation, imparts odor and aroma to beer¹¹⁰. Among the one to two hundred commercial hop varieties^{111,112}, there is wide variation in essential oil composition, encompassing aromas generally described as floral, citrus, spicy, herbal, grassy, woody, tropical, and fruity. These aromas are the result of a complex mixture of hundreds of compounds and the interactions between them.

Not all compounds present in fresh hop oil are retained in the final beer, however. Volatiles are lost during processing, transport, storage, and brewing. The monoterpene myrcene can account for as much as 70% of the fresh oil (in the Citra hop variety, for example) but is barely detectable in beer. On the other hand, hop-derived compounds not actually found in hop essential oil are present in beer, compounds that have been altered by yeast “tailoring” enzymes including reductases,

acetyltransferases, esterases, and glucosidases, or spontaneously generated by oxidation and hydrolysis reactions.

While linalool and geraniol biosynthesis is capable of conferring hop flavor to beer, the additional incorporation of sesquiterpene biosynthesis is likely to more fully reconstitute the flavor imparted by traditional hop additions. Sesquiterpenes are derived from the precursor farnesyl pyrophosphate (FPP), a 15-carbon molecule composed of three isoprene units produced through the mevalonate pathway. Yeast naturally produces FPP as a precursor in the ergosterol pathway, and previous terpene pathway engineering efforts in lab strains of *S. cerevisiae* have shown that yeast are capable of high sesquiterpene yields. High FPP synthase activity combined with a downregulation of *ERG9* (encoding squalene synthase) allows for high titers (hundreds of milligrams/L in shake flasks, which becomes grams/L in fermenters)^{113,114}. The sesquiterpenes caryophyllene and humulene can account for a combined 50% of hop essential oil (in the Fuggle hop variety, for example) and many other sesquiterpenes confer specific aromas to beer.

Terpene metabolism beyond terminal synthase enzymes can result in more complex hop-flavor bouquets and distinct flavor profiles, through controlled expression of genes responsible for enzymes that further modify terpenes. Beyond citronellol production through *OYE2* expression, one possible gene for expressing this type of activity is *ATF1*, which encodes an alcohol acetyltransferase known to modify a variety of flavor molecules, including terpenes, by the addition of acetyl groups. Many of the resulting esters, including linalyl, geranyl, and citronellyl acetate, are known to impart additional and/or more complex flavors to the resulting beer. *ATF1* has a homolog, *ATF2*, that exhibits similar activity. Both acetyltransferases are involved in the biosynthesis of other volatile esters, generally responsible for conferring fruity notes to beer; therefore expression of even a single gene will have a wide effect on the flavor of the resulting beer.

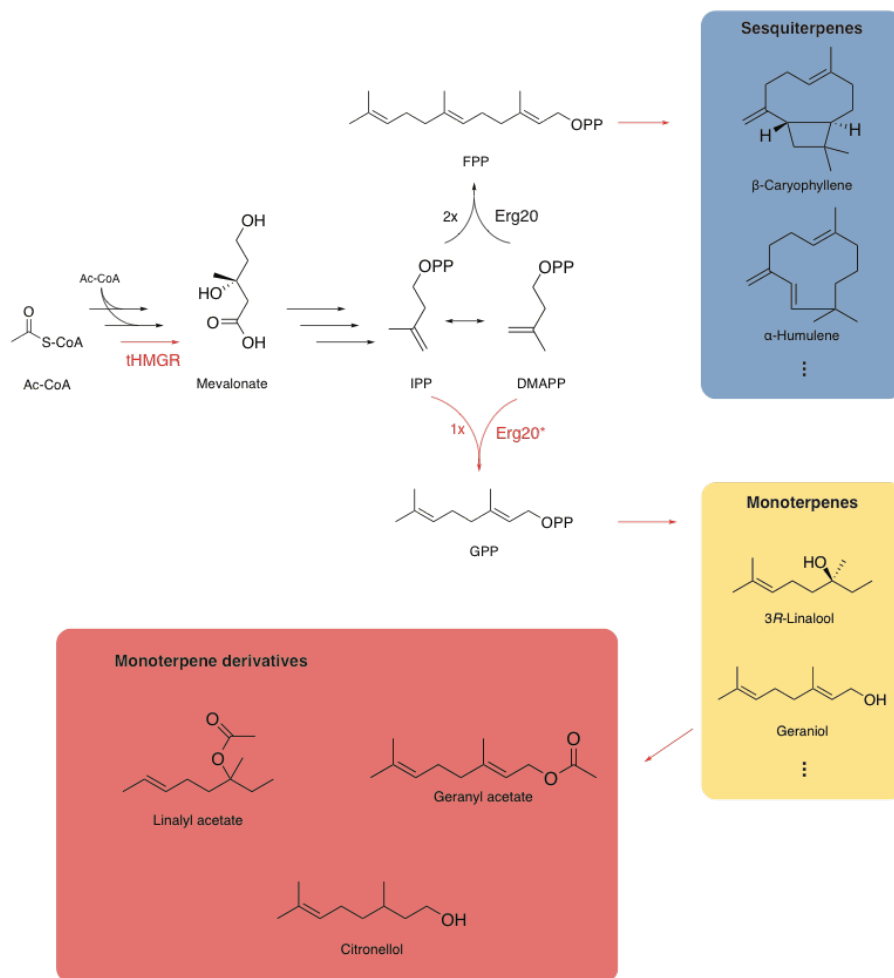


Figure 3.3. Biosynthesis of terpene molecules and terpene derivatives from yeast central carbon metabolism. Monoterpenes are derived from the precursor GPP, sesquiterpenes from FPP. Yeast-endogenous genes such as *OYE2* and *ATF1* affect conversion of terpenes to various derivatives (outlined by the red box), including alcohols and esters.

Beyond terpenes, a multitude of other flavor components in beer can be customized through yeast engineering. Heterologous expression of plant biosynthetic enzymes and overexpression of yeast tailoring enzymes will expand the repertoire of flavors made by brewer's yeasts and better reconstitute the full hop aroma. Tweaks to the expression of native yeast enzymes will enhance the aroma of hop-derived products and reduce the generation of off-flavors. Engineered strains provide an avenue for improvement and innovation of the brewing process by making it possible to generate new and distinctive flavors with consistency and reproducibility. Future strain engineering efforts can extend to other aspects of the brewing process, including the improvement of fermentation performance characteristics, to have a broader impact on the efficiency and the innovation of this centuries-old process.

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5. Appendices

5.1. Details of model development and performance evaluation

To establish a framework with which we could evaluate prospective genetic designs, we developed three mathematical models. Structured to contain a varying degree of prior biological knowledge, these models were used to predict the relationship between protein abundance and monoterpene production. Unstructured models, such as those used in deep-learning algorithms, are powerful in their capacity to capture complex biological relationships in large datasets. However, biologically-informed models may better capture quantitative relationships for qualitatively predictable biological phenomena in smaller datasets. Interestingly, the most biologically-informed model—based on Michaelis-Menten enzyme kinetics—best predicted actual monoterpene concentrations for the amount and type of data available.

The three models used in this work are a Gaussian regressor, a linear model, and a kinetic model. The Gaussian regressor model contains no biological intuition and is purely a sophisticated interpolation scheme. The linear model incorporates two pieces of biological intuition gleaned from the data and uses them to create a heuristic model that extrapolates well. The kinetic model is a detailed Michaelis-Menten-based model that uses in vitro kinetic coefficients from the literature where available. This model incorporates the most biological intuition and is derived almost strictly from the literature. Each model is described in more detail as follows:

The **Gaussian regressor model** is implemented in Scikit Learn and is the most 'off the shelf' of the models we used. Standard best practices for machine learning were used to validate and build the model. This model uses no biological intuition and is conceptually similar to an interpolation scheme. As this experiment was in vivo, the data may be influenced by more variables than what can intuitively be captured with biological intuition. Our thinking was that an unconstrained model may perform better than one constrained by a limited biological intuition. This seems to be the case with large data sets. With small data sets, however, models that include more biological intuition perform better. Like all of the models analyzed, it was validated using leave one out cross-validation on the first cycle of data.

The **linear model** incorporates the two design principles gleaned from the original data set and combines them together into a composite model that does well at predicting linalool and geraniol from the proteomics data. The first observation was that FPPS and tHMGR are both positively correlated with increasing total terpene titer. Second, the titer-normalized ratio of linalool to geraniol was predicted by the ratio of LIS to GES. As a result, we trained models to learn to predict total terpene titer from tHMGR and FPPS and the ratio of linalool to geraniol from the ratio of LIS to GES. Using all proteins, we can predict both total terpene titer and individual linalool and geraniol titers. Multiplying total titer by the percentage of linalool will yield total linalool titer. Similarly, multiplying total titer by the percentage of geraniol will yield total geraniol titer. Mathematically the predictions of these models can be found by:

$$\text{Total terpene production} = \alpha_1[tHMGR] + \alpha_2[FPPS] + \alpha_3$$

$$\text{Percent geraniol production} = \alpha_4[LIS] + \alpha_5[GES] + \alpha_6$$

$$\text{Percent linalool production} = \alpha_7[LIS] + \alpha_8[GES] + \alpha_9$$

$$[Linalool] = \text{Total terpene production} * \text{Percent linalool production}$$

$$[Geraniol] = \text{Total terpene production} * \text{Percent geraniol production}$$

The coefficients α_i above were calculated using linear machine learning models implemented in Scikit Learn. The composite model was validated using leave one out cross-validation.

The **kinetic model** developed here uses Michaelis-Menten kinetics together with information from the literature about the mevalonate pathway. This pathway has been heavily studied and so there are kinetic coefficients that were used as a starting point to parameterize the model. Our goal was to strike a balance between the number of free parameters in the model and the amount of detail. Increasing the number of free parameters (which come from additional sources of biological intuition) could lead to over-parameterizing the model. Ultimately, too many parameters with a small amount of data will make the model generalize poorly. Additionally, to avoid over-fitting, the model was cross-validated using a leave one out methodology. This model is subject to a few assumptions.

First, we assume that the intermediate metabolites do not cause feedback inhibition. This is reasonable because only HMGR appears to be feedback inhibited and this was addressed in strain engineering. A truncated HMGR that does not suffer from inhibition, tHMGR, was engineered into the host. Additionally, the goal is not to maximize production, but rather to hit a particular target to mimic the taste of hops in beer. As a result, feedback inhibition is much less of a concern than it would be if we were trying to maximize titers of linalool and geraniol. Second, we assume that IPP is an abundant enough reactant to not be included in the model. Third, tHMGR is assumed to be the rate-limiting step in a sequence of steps leading to GPP synthesis. To simplify the model, this entire linear sequence of steps was modeled by a single Michaelis-Menten kinetic equation that accurately describes the flux under tested conditions.

Both a native and a heterologous FPPS were present in the engineered host. This enzyme catalyzes two steps converting DMAPP and IPP into GPP, then GPP and IPP into FPP. The native enzyme has a quick conversion of GPP to FPP, whereas the engineered enzyme only slowly catalyzes that step. Since GPP is the precursor to both linalool and geraniol, this element is important to capture in the model. Together these assumptions and observations were used to craft the following system, which describes the pathway dynamics.

$$\begin{aligned}
\frac{d[DMAPP]}{dt} &= \frac{K_{cat6}[tHMGR][HMG-CoA]}{K_{m6}+[HMG-CoA]} - \frac{K_{cat0}[FPPS_1][DMAPP]}{K_{m0}+[DMAPP]} - \frac{K_{cat1}[FPPS_2][DMAPP]}{K_{m1}+[DMAPP]} \\
\frac{d[GPP]}{dt} &= \frac{K_{cat0}[FPPS_1][DMAPP]}{K_{m0}+[DMAPP]} + \frac{K_{cat1}[FPPS_2][DMAPP]}{K_{m1}+[DMAPP]} - \frac{K_{cat2}[FPPS_1][GPP]}{K_{m2}+[GPP]} \\
&\quad - \frac{K_{cat3}[FPPS_2][GPP]}{K_{m3}+[GPP]} - \frac{K_{cat4}[LIS][GPP]}{K_{m4}+[GPP]} - \frac{K_{cat5}[GES][GPP]}{K_{m5}+[GPP]} \\
\frac{d[Linalool]}{dt} &= \frac{K_{cat4}[LIS][GPP]}{K_{m4}+[GPP]} \\
\frac{d[Geraniol]}{dt} &= \frac{K_{cat5}[GES][GPP]}{K_{m5}+[GPP]}
\end{aligned}$$

The following components are modeled in the system: tHMGR, FPPS1, FPPS2, LIS, GES, IPP, DMAPP, GPP, FPP, linalool, geraniol. We assume the system has reached steady state so all nonterminal metabolite concentrations are constant. This means all metabolite derivatives except for linalool and geraniol are zero. Additionally, it is assumed that IPP concentrations are not limiting and it can be excluded from the dynamics of FPPS.

Free parameters are included to convert relative protein counts to absolute proteomics values. Additionally, a parameter determines the relative ratio between the endogenous FPPS and the engineered FPPS. Since FPPS is measured as a combination of the endogenous and engineered versions of the proteins we express $[FPPS_1]$ and $[FPPS_2]$ as functions of FPPS counts and the parameters α_0 and β . This is represented in the model by

$$[FPPS_2] = \alpha_0 (1 - \beta)[FPPS]$$

$$[FPPS_1] = \alpha_0 \beta [FPPS]$$

Kinetic coefficients were scraped from the literature. These served as starting points for the model parameters and were allowed to vary over an order of magnitude to improve model fit. We wanted to derive an optimization problem which would allow us to determine the production of linalool and geraniol from $[FPPS]$, $[LIS]$, $[GES]$, $[tHMGR]$. Given an existing data set, we found the parameters to fit the model. The free parameters include kinetic parameters bounded by the literature, conversion factors for relative to absolute proteomics, and a ratio parameter to fix the ratio of the engineered FPPS to the endogenous FPPS. These parameters are then optimized with respect to the training data set. After parameter optimization, the kinetic model performance is evaluated using leave one out cross-validation.

To evaluate the extent to which the second iteration genetic designs were targeted towards desired terpene biosynthesis levels, we compared simulated

monoterpene production for second iteration genetic designs with simulated production for randomly generated strains.

Univariate linear models were created which related genetic toolkit promoters (ranked 1-19) to protein concentrations for each modulated gene in the monoterpene pathway. The error residuals of the univariate linear models were used to compute noise for simulated protein levels. This was computed as:

$$\Omega \sim N(\mu_i, \sigma_i)$$

$$\omega \in \Omega$$

$$[protein]_i = f_i(p_{rank}) + \omega$$

where the function f_i corresponds to the model for protein i , and ω is a value generated at random from the error distribution Ω , corresponding to the model for protein i . The resulting protein concentrations were then used to compute terpene concentrations and noise was computed based on the error residual distribution from the kinetics-based model as follows:

$$\Xi \sim N_2(\mu, \Sigma_i)$$

$$\xi \in \Xi$$

$$[L, G] = g([tHMGR], [FPPS *], [LIS], [GES]) + \xi$$

where L and G are linalool and geraniol concentrations respectively. g is the mapping of the kinetics-based model from pathway protein concentrations to terpene concentrations. ξ is a random error residual drawn from the bivariate normal error residual distribution corresponding to the kinetics-based model. Using these equations, a single realization of phenotype predicted from genotype can be created.

To compare the predicted performance of second iteration strains to randomly generated strains, we created a pool of 25000 genotypes that were sampled from the 30 second iteration strains and a pool of 25000 genotypes that were generated by uniformly sampling promoter ranks between values 1 and 19 for each of the four promoter-modulated genes. The improvement of each model over random was evaluated and is presented in **Figure 2.11** and **Figure 5.1**.

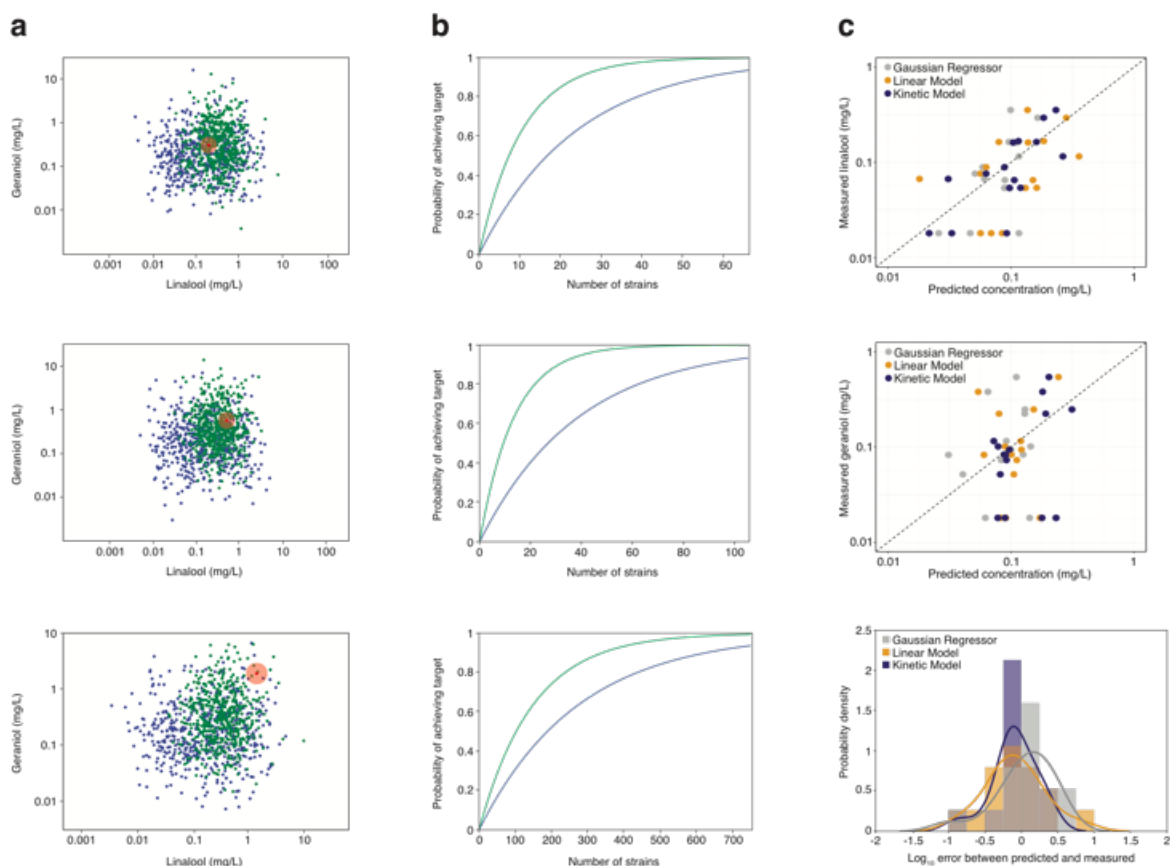


Figure 5.1. Predicting the extent of performance improvement for second iteration strains compared with randomly designed strains. **(a)** Simulated linalool and geraniol production corresponding to guided (blue) and naive (green) genetic designs. The red point represents the target beer and the surrounding red circle represents the area within 50% of the target concentrations. **(b)** Plots represent the number of strains expected to achieve target monoterpene concentrations for sampled commercial beers. Green corresponds to second iteration strains, blue corresponds to randomly designed strains. Target beers are (top to bottom) Sierra Nevada Pale Ale, Torpedo Extra IPA, and Hop Hunter IPA. In order to achieve a 90% chance of success at meeting the target terpene concentration, the number of strains from iteration two that would need to be constructed were predicted to be 26, 32, and 356 respectively. The predicted number of strains to construct are an improvement over random selection by 55.5% on average. **(c)** Relationship between predicted and measured monoterpene concentration of first iteration strains. Three models were used to describe the relationship between protein levels and monoterpene production. The Gaussian regressor model is an interpolation scheme with no biological intuition, the linear model is a composite of two linear regression models and captures biological insights gleaned from the data, and the kinetic model is structured according to principles of Michaelis-Menten enzyme kinetics (Online Methods, Supplementary Note 3). Error residuals of the three models are shown at the bottom. Residuals that are closely centered around zero correspond to a more accurate model fit.

5.2. Supplementary tables

		tHMGR	FPPS*	Linalool synthase	Geraniol synthase	Combined
Promoter and protein	Correlation	7.36E-01	8.16E-01	8.93E-01	8.66E-01	6.81E-01
	p-value	5.02E-04	3.60E-05	6.24E-07	3.34E-06	4.50E-11
Promoter and linalool	Correlation	2.73E-01	4.93E-01	7.35E-01	2.15E-01	3.95E-01
	p-value	2.74E-01	3.75E-02	5.05E-04	3.91E-01	5.94E-04
Promoter and geraniol	Correlation	5.94E-01	3.89E-01	1.81E-01	1.88E-01	2.87E-01
	p-value	9.39E-03	1.10E-01	4.73E-01	4.54E-01	1.46E-02
Promoter and total monoterpene	Correlation	3.42E-01	5.83E-01	5.80E-01	2.84E-01	4.01E-01
	p-value	1.65E-01	1.11E-02	1.17E-02	2.54E-01	4.75E-04
Protein and linalool	Correlation	5.12E-01	6.52E-01	8.21E-01	3.08E-01	5.35E-01
	p-value	2.99E-02	3.39E-03	2.98E-05	2.14E-01	1.31E-06
Protein and geraniol	Correlation	6.53E-01	4.26E-01	2.76E-01	2.24E-01	3.65E-01
	p-value	3.28E-03	7.76E-02	2.67E-01	3.72E-01	1.63E-03
Protein and total monoterpene	Correlation	5.94E-01	6.99E-01	6.72E-01	3.62E-01	5.46E-01
	p-value	9.36E-03	1.26E-03	2.25E-03	1.40E-01	7.11E-07

Supplementary Table 1

Correlations between strain characteristics

Model type		Mu	Sigma	Expected accuracy (90%)
Linear	Linalool	0.28	0.91	1.50
	Geraniol	0.31	1.21	1.99
	Total	0.20	0.93	1.53
Gaussian	Linalool	0.03	0.76	1.24
	Geraniol	0.16	1.25	2.06
	Total	-0.02	0.88	1.45
Kinetic	Linalool	0.17	0.65	1.07
	Geraniol	0.42	1.07	1.76
	Total	0.22	0.72	1.18

Supplementary Table 2

Statistics reflecting predictivity of mathematical modeling strategies

The moments of the error residuals are reported for each model in natural log space. Mu and sigma are the mean and standard deviation of the natural log error residuals. A zero value means zero model error. Expected accuracy is the size of the interval that captures 90% of the residual error in the model. In other words, the accuracy tells us that we expect 90% of the predictions to be within the provided window size in natural log error.

#	Equation
1	Total terpene production = $\alpha_1[\text{tHMGR}] + \alpha_2[\text{FPPS}] + \alpha_3$
2	% geraniol production = $\alpha_4[\text{LIS}] + \alpha_5[\text{GES}] + \alpha_6$
3	% linalool production = $\alpha_7[\text{LIS}] + \alpha_8[\text{GES}] + \alpha_9$
4	Linalool = Total terpene production % linalool production
5	Geraniol = Total terpene production % geraniol production

Supplementary Table 3

Equations used for implementing linear model

The model was structured to reflect the observations that, 1) abundance of FPPS and tHMGR are positively correlated with monoterpene production, and 2) relative abundance of t67-*Mc*LIS, and *Ob*GES correlated with ratio of monoterpenes linalool and geraniol.

Strain	Parent strain	Description	Reference
CEN.PK2-1D		<i>MATα ura3-52; trp1-289; leu2-3,112; his3Δ 1; MAL2-8C; SUC2</i>	Euroscarf
JBEI-14984	CEN.PK2-1D	<i>leu2::P_{GAL1}-ERG8-P_{GAL10}-ERG19-S.p.HIS3</i>	This study
JBEI-14985	JBEI-14984	<i>leu2::P_{GAL1}-ERG8-P_{GAL10}-ERG19-S.p.HIS3; ura3::P_{GAL1}-ERG20-F96WN127W-P_{GAL10}-IDI1-kanMX4</i>	This study
JBEI-14986	JBEI-14985	<i>leu2::P_{GAL1}-ERG8-P_{GAL10}-ERG19-S.p.HIS3; ura3::P_{GAL1}-ERG20-F96WN127W-P_{GAL10}-IDI1-kanMX4; HMG1::HphMX4-<i>Eα</i>MvaE-P_{GAL1/10}-<i>Eα</i>MvaS-P_{ADH1}-trnc530-HMG1</i>	This study
WLP001		California Ale Yeast	White Labs
JBEI-13510	WLP001	<i>ADE2Δ</i>	This study

Supplementary Table 4

Parental yeast strains used in this study

JBEI Number	Description	Marker	Ori	Related plasmid/strain
JBEI-16636	LEU2-HR-SkHis3MX4 P _{GAL1} -ERG8-T _{ERG8} P _{GAL10} -ERG19-T _{ERG19} LEU2-HR	Carb	ColE1	
JBEI-12291	URA3-HR-KanMX4 P _{GAL1} -ERG20-F96WN127W-T _{CYC1} P _{GAL10} -IDI1-T _{ADH1} URA3-HR	Carb	ColE1	JBEI-14985
JBEI-12289	HMG1-HR-HphMX4 <i>E</i> MvaE-T _{ADH1}	Carb	ColE1	JBEI-14986
JBEI-12285	P _{GAL1} - <i>E</i> MvaE(100nts) P _{GAL1} - <i>E</i> MvaS-T _{CYC1} P _{ADH1} -trnc530-HMG1	Carb	ColE1	JBEI-14986

Supplementary Table 5

Plasmids used for construction of GPP-hyper-producing strain

JBEI Number	Description	Marker	Ori	Related Figure
JBEI-10738	pESC-LEU2 (Invitrogen)	LEU2	2μ	Fig. 2.3d
JBEI-13024	pEntry_P _{GAL1} -sfGFP (pEntry_GoldenGate)	LEU2	2μ	Fig. 2.3d
JBEI-12279	P _{GAL1} -CbLIS	LEU2	2μ	Fig. 2.3d
JBEI-13019	P _{GAL1} -CbLIS-native	LEU2	2μ	Fig. 2.3d
JBEI-12313	P _{GAL1} -ApLIS	LEU2	2μ	Fig. 2.3d
JBEI-12298	P _{GAL1} -FaLIS	LEU2	2μ	Fig. 2.3d
JBEI-12304	P _{GAL1} -PflIS	LEU2	2μ	Fig. 2.3d
JBEI-13022	P _{GAL1} -McLIS	LEU2	2μ	Fig. 2.3d
JBEI-12334	P _{GAL1} -LeLIS	LEU2	2μ	Fig. 2.3d
JBEI-12299	P _{GAL1} -trnc26-ApLIS	LEU2	2μ	Fig. 2.3d
JBEI-12303	P _{GAL1} -trnc34-PflIS	LEU2	2μ	Fig. 2.3d
JBEI-13018	P _{GAL1} -trnc51-McLIS	LEU2	2μ	Fig. 2.3d
JBEI-12314	P _{GAL1} -trnc43-LeLIS	LEU2	2μ	Fig. 2.3d
JBEI-12306	P _{GAL1} -trnc19-FaLIS	LEU2	2μ	Fig. 2.3d
JBEI-12332	P _{GAL1} -trnc60-PflIS	LEU2	2μ	Fig. 2.3d
JBEI-13017	P _{GAL1} -trnc67-McLIS	LEU2	2μ	Fig. 2.3d
JBEI-14916	P _{GAL1} -CjGES	LEU2	2μ	Fig. 2.3d
JBEI-14921	P _{GAL1} -OeGES	LEU2	2μ	Fig. 2.3d
JBEI-14922	P _{GAL1} -PflGES	LEU2	2μ	Fig. 2.3d
JBEI-14923	P _{GAL1} -PdGES	LEU2	2μ	Fig. 2.3d
JBEI-14924	P _{GAL1} -VvGES	LEU2	2μ	Fig. 2.3d
JBEI-14917	P _{GAL1} -ObGES	LEU2	2μ	Fig. 2.3d
JBEI-14918	P _{GAL1} -trnc30-CjGES	LEU2	2μ	Fig. 2.3d

JBEI-14919	P _{GAL1} -trnc58-OeGES	LEU2	2μ	Fig. 2.3d
JBEI-14920	P _{GAL1} -trnc35-PfGES	LEU2	2μ	Fig. 2.3d
JBEI-14931	P _{GAL1} -trnc43-PdGES	LEU2	2μ	Fig. 2.3d
JBEI-14929	P _{GAL1} -trnc71-VvGES	LEU2	2μ	Fig. 2.3d
JBEI-14930	P _{GAL1} -trnc63-ObGES	LEU2	2μ	Fig. 2.3d
JBEI-14938	P _{GAL1} -trnc52-CjGES	LEU2	2μ	Fig. 2.3d
JBEI-14936	P _{GAL1} -trnc91-OeGES	LEU2	2μ	Fig. 2.3d
JBEI-14942	P _{GAL1} -trnc59-PfGES	LEU2	2μ	Fig. 2.3d
JBEI-14940	P _{GAL1} -trnc86-PdGES	LEU2	2μ	Fig. 2.3d
JBEI-14946	P _{GAL1} -trnc78-VvGES	LEU2	2μ	Fig. 2.3d
JBEI-14943	P _{GAL1} -trnc72-ObGES	LEU2	2μ	Fig. 2.3d

Supplementary Table 6

Plasmids used for heterologous protein expression in yeast

Species of origin: *Clarkia brewerii*, *Actinidia polygama*, *Fragaria x ananassa*, *Perilla frutescens*, *Mentha citrata*, *Lycopersicon esculentum*, *Citrus jambhiri*, *Olea europaea*, *Phyla dulcis*, *Vitis vinifera*, *Ocimum basilicum*

JBEI Number	Description	Marker	Ori	Related Figure
JBEI-13815	trnc530-HMG1	Cm	ColE1	Fig. 2.5d
JBEI-13814	ERG20-F96WN127W	Cm	ColE1	Fig. 2.5d
JBEI-13550	trnc67-McLIS	Cm	ColE1	Fig. 2.5d
JBEI-13546	<i>ObGES</i>	Cm	ColE1	Fig. 2.5d

Supplementary Table 7

Part plasmids generated in this study

JBEI Number	Description	Marker	Ori	Related Figure
JBEI-13898	pEntry-ADE2	KanR	ColE1	Fig. 2.5d
JBEI-14122	P _{TDH3} -trnc530-HMG1-T _{ENO1} P _{HHF1} -ERG20-F96WN127W-T _{SSA1} P _{RPL18b} -trnc67-McLIS-T _{ADH1} P _{HTB2} -ObGES-T _{PGK1}	KanR	ColE1	Fig. 2.5d
JBEI-14421	P _{ALD6} -trnc530-HMG1-T _{ENO1} P _{PGK1} -ERG20-F96WN127W-T _{SSA1} P _{HHF2} -trnc67-McLIS-T _{ADH1} P _{CCW12} -ObGES-T _{PGK1}	KanR	ColE1	Fig. 2.5d
JBEI-14423	P _{TDH3} -trnc530-HMG1-T _{ENO1} P _{PGK1} -ERG20-F96WN127W-T _{SSA1} P _{RPL18b} -trnc67-McLIS-T _{ADH1} P _{CCW12} -ObGES-T _{PGK1}	KanR	ColE1	Fig. 2.5d
JBEI-14424	P _{TDH3} -trnc530-HMG1-T _{ENO1} P _{PGK1} -ERG20-F96WN127W-T _{SSA1} P _{HHF2} -trnc67-McLIS-T _{ADH1} P _{CCW12} -ObGES-T _{PGK1}	KanR	ColE1	Fig. 2.5d
JBEI-14438	P _{TEF2} -trnc530-HMG1-T _{ENO1} P _{PGK1} -ERG20-F96WN127W-T _{SSA1} P _{HHF2} -trnc67-McLIS-T _{ADH1} P _{CCW12} -ObGES-T _{PGK1}	KanR	ColE1	Fig. 2.5d
JBEI-14458	P _{TDH3} -trnc530-HMG1-T _{ENO1} P _{PGK1} -ERG20-F96WN127W-T _{SSA1} P _{HHF2} -trnc67-McLIS-T _{ADH1} P _{HTB2} -ObGES-T _{PGK1}	KanR	ColE1	Fig. 2.5d
JBEI-14460	P _{TDH3} -trnc530-HMG1-T _{ENO1} P _{HHF1} -ERG20-F96WN127W-T _{SSA1} P _{HHF2} -trnc67-McLIS-T _{ADH1} P _{CCW12} -ObGES-T _{PGK1}	KanR	ColE1	Fig. 2.5d
JBEI-14925	P _{TEF2} -trnc530-HMG1-T _{ENO1} P _{PGK1} -ERG20-F96WN127W-T _{SSA1} P _{RPL18b} -trnc67-McLIS-T _{ADH1} P _{RNR1} -ObGES-T _{PGK1}	KanR	ColE1	Fig. 2.5d
JBEI-14926	P _{TEF2} -trnc530-HMG1-T _{ENO1} P _{PGK1} -ERG20-F96WN127W-T _{SSA1} P _{HHF2} -trnc67-McLIS-T _{ADH1} P _{RNR1} -ObGES-T _{PGK1}	KanR	ColE1	Fig. 2.5d

JBEI-14928	P _{TDH3} -trnc530-HMG1-T _{ENO1} P _{PAB1} -ERG20-F96WN127W-T _{SSA1} P _{RNR2} -trnc67-McLIS-T _{ADH1} P _{HTB2} -ObGES-T _{PGK1}	KanR	ColE1	Fig. 2.5d
JBEI-14932	P _{TDH3} -trnc530-HMG1-T _{ENO1} P _{PAB1} -ERG20-F96WN127W-T _{SSA1} P _{RPL18b} -trnc67-McLIS-T _{ADH1} P _{HTB2} -ObGES-T _{PGK1}	KanR	ColE1	Fig. 2.5d
JBEI-14935	P _{TEF2} -trnc530-HMG1-T _{ENO1} P _{PAB1} -ERG20-F96WN127W-T _{SSA1} P _{RPL18b} -trnc67-McLIS-T _{ADH1} P _{HTB2} -ObGES-T _{PGK1}	KanR	ColE1	Fig. 2.5d
JBEI-14937	P _{ALD6} -trnc530-HMG1-T _{ENO1} P _{HHF1} -ERG20-F96WN127W-T _{SSA1} P _{RPL18b} -trnc67-McLIS-T _{ADH1} P _{CCW12} -ObGES-T _{PGK1}	KanR	ColE1	Fig. 2.5d
JBEI-14945	P _{TEF2} -trnc530-HMG1-T _{ENO1} P _{HHF1} -ERG20-F96WN127W-T _{SSA1} P _{RNR2} -trnc67-McLIS-T _{ADH1} P _{CCW12} -ObGES-T _{PGK1}	KanR	ColE1	Fig. 2.5d
JBEI-14948	P _{TEF2} -trnc530-HMG1-T _{ENO1} P _{HHF1} -ERG20-F96WN127W-T _{SSA1} P _{RNR2} -trnc67-McLIS-T _{ADH1} P _{HTB2} -ObGES-T _{PGK1}	KanR	ColE1	Fig. 2.5d
JBEI-14949	P _{TDH3} -trnc530-HMG1-T _{ENO1} P _{HHF1} -ERG20-F96WN127W-T _{SSA1} P _{RPL18b} -trnc67-McLIS-T _{ADH1} P _{RNR1} -ObGES-T _{PGK1}	KanR	ColE1	Fig. 2.5d
JBEI-14950	P _{TEF2} -trnc530-HMG1-T _{ENO1} P _{HHF1} -ERG20-F96WN127W-T _{SSA1} P _{RNR2} -trnc67-McLIS-T _{ADH1} P _{RNR1} -ObGES-T _{PGK1}	KanR	ColE1	Fig. 2.5d

Supplementary Table 8

Pathway plasmids used for construction of initial test set of industrial brewing strains

JBEI Number	Description	Marker	Ori
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JBEI-13898	pENTRY-ADE2	KanR	ColE1
JBEI-16632	P _{PGK1} -trnc530-HMG1-T _{ENO1} P _{TDH3} -ERG20-F96WN127W-T _{SSA1} P _{RPL18b} -trnc67-McLIS-T _{ADH1} P _{HHF2} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16633	P _{PGK1} -trnc530-HMG1-T _{ENO1} P _{CCW12} -ERG20-F96WN127W-T _{SSA1} P _{TEF2} -trnc67-McLIS-T _{ADH1} P _{HHF2} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16634	P _{HHF2} -trnc530-HMG1-T _{ENO1} P _{CCW12} -ERG20-F96WN127W-T _{SSA1} P _{TEF2} -trnc67-McLIS-T _{ADH1} P _{TDH3} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16635	P _{PGK1} -trnc530-HMG1-T _{ENO1} P _{HHF2} -ERG20-F96WN127W-T _{SSA1} P _{HTB2} -trnc67-McLIS-T _{ADH1} P _{CCW12} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16637	P _{TDH3} -trnc530-HMG1-T _{ENO1} P _{HHF1} -ERG20-F96WN127W-T _{SSA1} P _{TEF1} -trnc67-McLIS-T _{ADH1} P _{HHF2} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16638	P _{TDH3} -trnc530-HMG1-T _{ENO1} P _{HHF2} -ERG20-F96WN127W-T _{SSA1} P _{HTB2} -trnc67-McLIS-T _{ADH1} P _{TEF2} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16639	P _{TDH3} -trnc530-HMG1-T _{ENO1} P _{HHF1} -ERG20-F96WN127W-T _{SSA1} P _{RPL18b} -trnc67-McLIS-T _{ADH1} P _{CCW12} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16640	P _{CCW12} -trnc530-HMG1-T _{ENO1} P _{PGK1} -ERG20-F96WN127W-T _{SSA1} P _{TEF2} -trnc67-McLIS-T _{ADH1} P _{HHF2} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16641	P _{HHF2} -trnc530-HMG1-T _{ENO1} P _{TDH3} -ERG20-F96WN127W-T _{SSA1} P _{RPL18b} -trnc67-McLIS-T _{ADH1} P _{CCW12} -ObGES-T _{PGK1}	KanR	ColE1

JBEI-16642	P _{HHF2} -trnc530-HMG1-T _{ENO1} P _{TEF1} -ERG20-F96WN127W-T _{SSA1} P _{HTB2} -trnc67-McLIS-T _{ADH1} P _{TDH3} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16643	P _{TDH3} -trnc530-HMG1-T _{ENO1} P _{HHF1} -ERG20-F96WN127W-T _{SSA1} P _{TEF2} -trnc67-McLIS-T _{ADH1} P _{HHF2} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16644	P _{TDH3} -trnc530-HMG1-T _{ENO1} P _{CCW12} -ERG20-F96WN127W-T _{SSA1} P _{TEF1} -trnc67-McLIS-T _{ADH1} P _{TEF2} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16646	P _{TEF2} -trnc530-HMG1-T _{ENO1} P _{CCW12} -ERG20-F96WN127W-T _{SSA1} P _{TEF1} -trnc67-McLIS-T _{ADH1} P _{TDH3} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16647	P _{TEF2} -trnc530-HMG1-T _{ENO1} P _{CCW12} -ERG20-F96WN127W-T _{SSA1} P _{RPL18b} -trnc67-McLIS-T _{ADH1} P _{TDH3} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16648	P _{TEF2} -trnc530-HMG1-T _{ENO1} P _{HHF2} -ERG20-F96WN127W-T _{SSA1} P _{RPL18b} -trnc67-McLIS-T _{ADH1} P _{TDH3} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16649	P _{TEF2} -trnc530-HMG1-T _{ENO1} P _{TEF1} -ERG20-F96WN127W-T _{SSA1} P _{HTB2} -trnc67-McLIS-T _{ADH1} P _{HHF2} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16650	P _{TEF2} -trnc530-HMG1-T _{ENO1} P _{TEF1} -ERG20-F96WN127W-T _{SSA1} P _{HTB2} -trnc67-McLIS-T _{ADH1} P _{TDH3} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16651	P _{TEF2} -trnc530-HMG1-T _{ENO1} P _{PGK1} -ERG20-F96WN127W-T _{SSA1} P _{TEF1} -trnc67-McLIS-T _{ADH1} P _{HHF2} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16653	P _{CCW12} -trnc530-HMG1-T _{ENO1} P _{TEF1} -ERG20-F96WN127W-T _{SSA1} P _{HTB2} -trnc67-McLIS-T _{ADH1} P _{HHF2} -ObGES-T _{PGK1}	KanR	ColE1

JBEI-16655	P _{CCW12} -trnc530-HMG1-T _{ENO1} P _{HHF1} -ERG20-F96WN127W-T _{SSA1} P _{RPL18b} -trnc67-McLIS-T _{ADH1} P _{HHF2} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16656	P _{CCW12} -trnc530-HMG1-T _{ENO1} P _{PGK1} -ERG20-F96WN127W-T _{SSA1} P _{TEF1} -trnc67-McLIS-T _{ADH1} P _{TDH3} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16657	P _{TDH3} -trnc530-HMG1-T _{ENO1} P _{PAB1} -ERG20-F96WN127W-T _{SSA1} P _{HHF2} -trnc67-McLIS-T _{ADH1} P _{CCW12} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16658	P _{TEF2} -trnc530-HMG1-T _{ENO1} P _{HHF1} -ERG20-F96WN127W-T _{SSA1} P _{HHF2} -trnc67-McLIS-T _{ADH1} P _{CCW12} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16659	P _{TDH3} -trnc530-HMG1-T _{ENO1} P _{PGK1} -ERG20-F96WN127W-T _{SSA1} P _{HHF2} -trnc67-McLIS-T _{ADH1} P _{RNR1} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16660	P _{TEF2} -trnc530-HMG1-T _{ENO1} P _{HHF2} -ERG20-F96WN127W-T _{SSA1} P _{TEF1} -trnc67-McLIS-T _{ADH1} P _{CCW12} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16662	P _{PGK1} -trnc530-HMG1-T _{ENO1} P _{HHF1} -ERG20-F96WN127W-T _{SSA1} P _{TEF1} -trnc67-McLIS-T _{ADH1} P _{TEF2} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16665	P _{PGK1} -trnc530-HMG1-T _{ENO1} P _{TEF1} -ERG20-F96WN127W-T _{SSA1} P _{CCW12} -trnc67-McLIS-T _{ADH1} P _{HHF2} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16675	P _{HHF2} -trnc530-HMG1-T _{ENO1} P _{CCW12} -ERG20-F96WN127W-T _{SSA1} P _{RPL18b} -trnc67-McLIS-T _{ADH1} P _{TEF2} -ObGES-T _{PGK1}	KanR	ColE1
JBEI-16678	P _{CCW12} -trnc530-HMG1-T _{ENO1} P _{HHF1} -ERG20-F96WN127W-T _{SSA1} P _{TEF1} -trnc67-McLIS-T _{ADH1} P _{TDH3} -ObGES-T _{PGK1}	KanR	ColE1

JBEI-16683	$P_{\text{HHF2}}\text{-trnc530-HMG1-T}_{\text{ENO1}}$ $P_{\text{TDH3}}\text{-ERG20-F96WN127W-T}_{\text{SSA1}}$ $P_{\text{TEF2}}\text{-trnc67-McLIS-T}_{\text{ADH1}}$ $P_{\text{CCW12}}\text{-ObGES-T}_{\text{PGK1}}$	KanR	ColE1
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Supplementary Table 9

Pathway plasmids used for construction of second set of industrial brewing strains

Strain	Linalool (mg/L)	Standard deviation (Linalool)	Geraniol (mg/L)	Standard deviation (Geraniol)
JBEI-14953	3.843	0.556	0.503	0.086
JBEI-14954	0.162	0.012	0.543	0.062
JBEI-14955	0.115	0.012	0.248	0.061
JBEI-14968	0.067	0.032	0.380	0.040
JBEI-14969	0.294	0.132	0.101	0.038
JBEI-14970	0.338	0.028	0.405	0.031
JBEI-14971	0.089	0.020	0.083	0.021
JBEI-14972	0.164	0.022	0.224	0.027
JBEI-14973	0.021	0.005	0.048	0.005
JBEI-14974	0.054	0.004	0.083	0.011
JBEI-14975	0.054	0.019	0.073	0.030
JBEI-14976	0.065	0.002	0.094	0.002
JBEI-14983	0.004	0.001	0.008	0.008
JBEI-14988	0.001	0.000	0.011	0.019
JBEI-14989	0.001	0.000	0.052	0.025
JBEI-14990	0.076	0.027	0.003	0.000
JBEI-15262	0.167	0.059	0.115	0.043
JBEI-15263	0.354	0.030	0.003	0.000
JBEI-16095	0.274	0.013	0.090	0.046
JBEI-16096	0.040	0.001	0.030	0.007
JBEI-16097	0.029	0.020	0.069	0.059
JBEI-16098	0.261	0.015	0.433	0.054
JBEI-16099	0.463	0.269	0.149	0.215
JBEI-16598	0.478	0.019	0.369	0.022
JBEI-16599	0.469	0.024	0.461	0.010
JBEI-16619	1.011	0.051	1.453	0.060
JBEI-16621	0.626	0.041	0.493	0.035
JBEI-16645	0.267	0.032	0.036	0.006
JBEI-16652	0.104	0.068	0.480	0.018
JBEI-16654	0.048	0.053	0.005	0.008
JBEI-16661	0.022	0.002	0.001	0.000
JBEI-16663	0.633	0.024	0.528	0.062
JBEI-16664	0.162	0.150	0.002	0.000
JBEI-16666	0.248	0.033	0.571	0.089
JBEI-16667	3.584	1.036	1.428	0.379
JBEI-16668	0.190	0.011	0.001	0.000
JBEI-16669	0.117	0.074	0.444	0.108
JBEI-16670	0.112	0.003	0.001	0.000

JBEI-16671	1.418	0.034	0.043	0.001
JBEI-16672	0.595	0.083	0.001	0.000
JBEI-16673	0.246	0.262	0.214	0.123
JBEI-16674	0.774	0.036	0.042	0.003
JBEI-16676	0.295	0.030	0.704	0.018
JBEI-16677	0.614	0.015	0.388	0.029
JBEI-16679	0.558	0.018	0.209	0.110
JBEI-16680	0.506	0.043	0.856	0.068
JBEI-16681	0.193	0.019	0.573	0.075
JBEI-16682	0.882	0.073	0.173	0.089
Iteration 1 Centroid	0.326	0.053	0.165	0.028
Iteration 2 Centroid	0.507	0.085	0.341	0.056

Supplementary Table 10

Monoterpene concentrations after fermentation as measured by GCMS

Strain	Maltotriose (g/L)	Maltose (g/L)	Glucose (g/L)	Ethanol (g/L)
JBEI-14953	6.618	19.057	0.0047	14.348
JBEI-14954	2.853	3.463	0.1124	26.659
JBEI-14955	0.232	0.795	0.0047	23.009
JBEI-14968	2.335	2.841	0.0360	15.534
JBEI-14969	0.905	2.081	0.0138	23.174
JBEI-14970	7.339	21.372	0.0149	10.633
JBEI-14971	0.009	0.840	0.0047	20.583
JBEI-14972	0.595	1.026	0.0047	24.260
JBEI-14973	6.898	19.588	0.1330	11.125
JBEI-14974	2.869	2.791	0.0047	24.060
JBEI-14975	2.591	2.890	0.0047	22.274
JBEI-14976	1.202	2.925	0.0047	25.090
JBEI-14983	3.684	4.091	0.0123	22.964
JBEI-14988	0.815	1.177	0.1102	25.436
JBEI-14989	0.009	0.169	0.0148	26.118
JBEI-14990	1.884	2.118	0.0047	22.028
JBEI-15262	4.227	5.019	0.0125	21.910
JBEI-15263	0.009	1.072	0.0177	26.931
JBEI-16095	3.354	1.965	0.0003	33.710
JBEI-16096	3.278	1.790	0.0003	32.416
JBEI-16097	3.797	2.574	0.0003	30.510
JBEI-16098	3.545	2.319	0.0003	32.954
JBEI-16099	5.482	7.381	0.0003	30.388
JBEI-16598	3.170	1.671	0.0003	35.054
JBEI-16599	3.591	2.551	0.0003	31.833
JBEI-16619	3.439	2.129	0.0003	33.768
JBEI-16621	2.762	0.820	0.0003	34.237
JBEI-16645	3.123	1.648	0.0003	31.893
JBEI-16652	3.656	2.353	0.0003	33.348
JBEI-16654	8.422	23.631	0.0003	18.197
JBEI-16661	3.157	1.600	0.0003	33.361
JBEI-16663	3.172	1.779	0.0003	31.887
JBEI-16664	3.311	2.148	0.0003	30.959
JBEI-16666	3.083	1.693	0.0003	33.701
JBEI-16667	4.310	1.498	0.0003	34.826
JBEI-16668	4.965	6.231	0.0003	29.774
JBEI-16669	3.291	1.855	0.0003	35.041
JBEI-16670	2.873	1.362	0.0003	34.791
JBEI-16671	3.598	2.721	0.0003	34.560

JBEI-16672	3.493	2.585	0.0003	31.437
JBEI-16673	4.197	3.768	0.0003	33.124
JBEI-16674	3.683	2.429	0.0003	33.242
JBEI-16676	3.188	1.807	0.0003	34.068
JBEI-16677	4.546	3.870	0.0003	32.554
JBEI-16679	2.997	1.646	0.0003	33.865
JBEI-16680	4.062	3.097	0.0003	32.429
JBEI-16681	3.700	3.024	0.0003	31.154
JBEI-16682	3.536	2.287	0.0003	34.449

Supplementary Table 11

Fermentable sugars remaining and ethanol produced after fermentation as measured by HPLC

Strain	tHMGR	FPPS*	Linalool synthase	Geraniol synthase
JBEI-14953	46294.300	61749.710	7402.689	694.593
JBEI-14954	16710.980	30271.121	1362.031	2080.742
JBEI-14955	13453.780	26290.896	3551.335	3696.098
JBEI-14968	5795.770	9286.386	336.350	1745.693
JBEI-14969	8778.641	22922.219	2464.120	402.745
JBEI-14970	14127.300	32855.923	2958.891	3091.251
JBEI-14971	6758.793	9092.046	1147.474	417.553
JBEI-14972	9571.769	10945.530	2142.471	1906.866
JBEI-14973	19389.671	10879.197	568.186	230.264
JBEI-14974	15704.779	8995.311	1574.220	377.276
JBEI-14975	9319.016	14119.950	1269.566	438.467
JBEI-14976	11020.566	14877.566	1391.894	512.985
JBEI-14983	2983.832	14838.114	1185.394	1718.830
JBEI-14988	5754.331	9356.943	365.620	237.955
JBEI-14989	6356.994	11020.338	208.094	465.921
JBEI-14990	8227.666	8338.383	777.102	405.419
JBEI-15262	9743.995	18081.904	1503.451	295.152
JBEI-15263	2900.501	23202.188	3124.680	2512.464
JBEI-16095	20474.100	17711.137	8545.443	622.860
JBEI-16096	19124.540	19994.175	2997.740	9172.155
JBEI-16097	18091.790	18404.273	2563.367	3787.273
JBEI-16098	17514.190	31567.677	1916.223	2496.267
JBEI-16099	17369.357	18501.317	9925.830	3490.040
JBEI-16598	19610.067	37980.823	7472.970	3524.570
JBEI-16599	13396.880	57354.287	12314.740	5166.850
JBEI-16619	16206.527	45602.563	2782.870	3451.343
JBEI-16621	24364.443	29458.623	11062.143	2999.473
JBEI-16645	19822.733	17402.283	10495.493	2806.700
JBEI-16652	26006.783	28662.457	1296.853	1501.493
JBEI-16654	20618.583	18952.213	3097.653	1883.330
JBEI-16661	17359.100	27385.927	3907.510	4477.920
JBEI-16663	16625.343	14240.707	6036.790	5295.740
JBEI-16664	52963.143	23671.387	13908.027	11244.240
JBEI-16666	10348.877	30232.160	3044.643	4665.903
JBEI-16667	14089.147	31531.993	6465.207	802.990
JBEI-16668	18744.913	15292.113	8482.073	1011.540
JBEI-16669	13195.290	18793.995	1910.315	7660.925
JBEI-16670	20967.203	19471.620	10985.173	2185.243
JBEI-16671	18254.780	25980.673	7154.360	2727.577

JBEI-16672	51190.310	45493.357	12366.913	965.126
JBEI-16673	11475.077	17722.067	1250.893	550.480
JBEI-16674	14500.283	41299.713	8351.270	1704.693
JBEI-16676	11254.683	61582.137	3897.487	5965.893
JBEI-16677	10083.613	12135.973	6239.280	2339.720
JBEI-16679	13516.547	60292.777	13508.283	3775.027
JBEI-16680	23179.263	54941.557	2750.157	3207.057
JBEI-16681	13192.357	61378.780	3676.633	7408.330
JBEI-16682	21734.797	38277.207	9907.590	1267.464

Supplementary Table 12

Protein abundance (total protein area)

Sample	Apparent extract (%m/m)	Real extract (%m/m)	Alcohol (%V/V)	Density (g/cm ³)	Specific gravity	RDF (%)
WLP001	2.13	3.96	5.03	1.0065	1.00831	67.3
JBEI-14971	2.7	4.38	4.64	1.0087	1.01051	63.14
JBEI-16669	2.92	4.59	4.58	1.0096	1.01142	61.72
JBEI-16652	4.22	5.61	3.84	1.0147	1.01653	52.39

Supplementary Table 13

Alcolyzer measurements of industrial fermentations (first set)

Sample	Apparent extract (%m/m)	Real extract (%m/m)	Alcohol (%V/V)	Density (g/cm ³)	Specific gravity	RDF (%)
WLP001	2.19	3.94	4.81	1.0067	1.00851	66.44
JBEI-16652	3.71	5.13	3.89	1.0127	1.01452	54.96
WLP001 WA dry hop	2.26	3.89	4.45	1.007	1.00881	64.97
WLP001 ID dry hop	2.31	4.01	4.67	1.0072	1.00901	65.32

Supplementary Table 14

Alcolyzer measurements of industrial fermentations (second set)