#798

BERKELEY BREWING SCIENCE

Berkeley Brewing Science
2332 4th Street, Suite E
Berkeley, CA 94710

June 20, 2018

GRAS Notification Program
Office of Food Additive Safety, HFS-200
5001 Campus Drive
College Park, MD 20740-3835

Dear Sir or Madam,

We are hereby submitting one hard copy and one electronic copy of a new generally recognized as safe (GRAS) notification, in accordance with Title 21 C.F.R. §170.30, for Berkeley Brewing Science's *Saccharomyces cerevisiae* strain yBBS002. The electronic copy is provided on a virus-free CD, and is an exact copy of the paper submission. Berkeley Brewing Science has determined through scientific procedures the the yBBS002 strain is generally recognized as safe for use in the brewing industry to impart hoppy flavors to beer during fermentation.

Our most recent pre-submission meeting with the FDA was on June 11, 2018.

Please do not hesitate to contact me at any time by email at charles@bbsbeer.com to discuss details or to request supplemental information as needed.

Thank you for your time and your consideration.

(b) (6)

Charles Denby, Ph.D.
Berkeley Brewing Science

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE (Subpart E of Part 170)

Form Approved: OMB No. 0910-0342; Expiration Date: 09/30/2019
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration,5001 Campus Drive, College Park, MD 20740-3835.

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

☒ New ☐ Amendment to GRN No. _____ ☐ Supplement to GRN No. _____

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3 Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd)*: 2018/06/11

4 For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd)*: _____

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier	Name of Contact Person: Charles Denby		Position or Title: CEO	
	Organization *(if applicable)*: Berkeley Brewing Science, Inc.			
	Mailing Address *(number and street)*: 2332 4th Street SpaceE			
City: Berkeley	State or Province: California	Zip Code/Postal Code: 94710	Country: United States of America	
Telephone Number: 206-799-2668	Fax Number:	E-Mail Address: charles@bbsbeer.com		
1b. Agent or Attorney *(if applicable)*	Name of Contact Person:		Position or Title:	
	Organization *(if applicable)*:			
	Mailing Address *(number and street)*:			
City:	State or Province:	Zip Code/Postal Code:	Country:	
Telephone Number:	Fax Number:	E-Mail Address:		

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

Saccharomyces cerevisiaestrain yBBS002

2. Submission Format: *(Check appropriate box(es))*

☒ Electronic Submission Gateway

☐ Electronic files on physical media

☐ Paper

If applicable give number and type of physical media

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in CFSAN's files? *(Checkone)*

☐ Yes *(Proceed to Item 5)* ☒ No *(Proceed to Item 6)*

5. The submission incorporates information from a previous submission to FDA as indicated below *(Check all that apply)*

☐ a) GRAS Notice No. GRN _____

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☐ e) Other or Additional *(describe or enter information as above)* _____

6. Statutory basis for conclusions of GRAS status *(Check one)*

☒ Scientific procedures *(21 CFR 170.30(a) and (b))* ☐ Experience based on common use in food *(21 CFR 170.30(a) and (c))*

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? (see 21 CFR 170.225(c)(8))

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

SECTION D – INTENDED USE

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

Strain yBBS002 is intended for useasastarter culture for brewing wort fermentation, to impart hoppy flavors to beer during fermentation.The strain produces final concentrations of target flavor molecules in beer that aresimilar to those achieved with traditional hopping, without the use of hops.We recommend that brewers useapproximately 1 million cells per milliliter of wort per degree Plato, asisstandard industrial practice.

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☒ No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☐ No , you ask us to exclude trade secrets from the information FDA will send to FSIS.

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)

☒ PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).

☒ PART 3 of a GRAS notice: Dietary exposure (170.235).

☒ PART 4 of a GRAS notice: Self-limiting levels of use (170.240).

☒ PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).

☒ PART 6 of a GRAS notice: Narrative (170.250).

☒ PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☐ Yes ☒ No

Did you include this other information in the list attachr

☐ Yes ☐ No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Berkeley Brewing Science, Inc.

(name of notifier)

has concluded that the intended use*(s)* of Saccharomyces cerevisiaestrain yBBS002

(name of notified substance)

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. Berkeley Brewing Science, Inc. agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.

(name of notifier)

2332 4th Street SpaceE,Berkeley CA 94710

(address of notifier or other location)

The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance.The notifying party certifies that the information provided herein is accurate and complete to the best or his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
	Charles Denby,CEO	06/19/2018

SECTION G – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
1	Insert Clear Cover_Letter	Administrative
2	Insert Clear Part_1_S_cerevisiae_yBBS002	Administrative
3	Insert Clear GRAS_Notification_S_cerevisiae_yBBS002	Submission
4	Insert Clear Supporting_data	Administrative
	Insert Clear	
	Insert Clear	
	Insert Clear	
	Insert Clear	
	Insert Clear	

Add Continuation Page

OMB Statement: Public reporting burden for this collection of information is estimated to average 170 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services,Food and Drug Administration, Office of Chief Information Officer, PRAStaff@fda.hhs.gov. (Please do NOT return the form to this address.). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.

GRAS notice for *Saccharomyces cerevisiae* strain yBBS002

PREPARED FOR:
Office of Food Additive Safety (FHS-200)
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740-3835

DATE:
19 June 2018

PART 1: SIGNED STATEMENTS AND CERTIFICATION

In accordance with Title 21 C.F.R. §170.30, Berkeley Brewing Science, Inc. hereby informs the United States (U.S.) Food and Drug Administration (FDA) of the view that its *Saccharomyces cerevisiae* strain yBBS002 is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on its conclusion that the notified substance is Generally Recognized as Safe (GRAS) under the conditions of its intended use described in Part 1.3 below. In addition, as a responsible official of Berkeley Brewing Science, Inc., the undersigned hereby certifies that all data and information presented in this notice constitutes a complete, representative, and balanced submission, and which considered all unfavorable as well as favorable information known to Berkeley Brewing Science, Inc. and pertinent to the evaluation of the safety and GRAS status of strain yBBS002, as described herein.

No information used in this part of this notification is trade secret or confidential commercial information. In accordance with the requirements outlined in 21 CFR 170, Subpart E of the final rule, the following information is included with this exemption claim:

1.1. Name and address of the notifier

Berkeley Brewing Science, Inc.
2332 4th Street, Suite E
Berkeley, CA 94710

Project Director: Charles Denby
Scientific Manager: Rachel Li

1.2. Common or usual name of the notified substance

The subject of the notice is a brewing yeast obtained from a *Saccharomyces cerevisiae* strain carrying four recombinant genes encoding HMG-CoA reductase and FPP synthase derived from *Saccharomyces cerevisiae* (baker's and brewing yeast), a linalool synthase derived from *Mentha citrata* (mint), and a geraniol synthase derived from *Ocimum basilicum* (basil). This novel yeast strain is called yBBS002.

1.3. Intended conditions of use

Brewer's yeast developed by Berkeley Brewing Science (BBS) produces the primary flavor determinants of hops during fermentation, thereby reducing the need to use hops during beer production (Figure 1). Specifically, BBS introduced genes into the brewer's yeast genome that lead to biosynthesis of linalool, geraniol, and citronellol, three flavor molecules that together impart Cascade hop flavor to beer[1, 2]. Cascade is the most popular cultivar of hops in American Craft brewing. By engineering

yeast to biosynthesize flavor molecules, BBS will reduce the hops used in Craft brewing, thereby shrinking the environmental footprint of the brewing process. In addition, engineered yeast will address the concerns shared by many brewers of the lack of consistency in flavor.

To address these challenges, we propose the use of a genetically enhanced strain of *Saccharomyces cerevisiae* named yBBS002 to impart hoppy flavors to beer during fermentation. The modified strain contains the *Saccharomyces cerevisiae* HMG-CoA reductase gene (HMG1), the *Saccharomyces cerevisiae* FPP synthase gene (ERG20), the *Mentha citrata* linalool synthase gene, and the *Ocimum basilicum* geraniol synthase gene. Therefore all genetic donors are derived from brewer's yeast or edible plants that are commonly used in the US food supply. This strain produces final concentrations of target flavor molecules in beer that are similar to those achieved with traditional hopping, without the use of hops. For detailed description of integrated genetic material see Section 2.3.2.

The yBBS002 liquid yeast is intended for use as a starter culture for brewing wort fermentation, in accordance with Good Manufacturing Practices (GMP). Like all liquid brewing yeast, the liquid yeast obtained with the yBBS002 strain is used to perform alcoholic fermentation, thus transforming brewing wort into beer. Simultaneously the yBBS002 yeast cells produce hop flavor/aroma compounds, normally imparted to beer through the processes of dry hopping. Many beer styles are dry-hopped and may benefit from the use of BBS' yBBS002 proprietary yeast strain.

1.4. Basis for the GRAS determination

Berkeley Brewing Science, Inc. has determined, through scientific analysis, that the yeast yBBS002 is "Generally Regarded as Safe" (GRAS) for use as a starter culture in brewing fermentation. Based on the scientific data concerning the yBBS002 strain, the company has found that yBBS002 is substantially equivalent to the host strain from which it was derived, except for the introduction of a metabolic pathway producing monoterpene alcohols, which are ordinarily present in beer at comparable concentrations as a result of hop addition. Specifically, the genetic insertions are all derived from *S. cerevisiae* and the edible plants *Mentha citrata* and *Ocimum basilicum*.

The newly incorporated genetic material was introduced at a single locus, and DNA diagnostic methods confirmed the presence of the on-target integration event, as well as the absence of major genetic rearrangements. Further, the extreme similarity between beer composition resulting from parent and yBBS002 fermentation was illustrated by comparative metabolomics. This notice includes a safety evaluation of the host microorganism, of the foreign genetic material used to construct the modified yeast strain, of the modified yeast strain, and of the beer produced using the modified yeast strain.

1.5. Availability to FDA of data and information that are the basis of determination

Berkeley Brewing Science, Inc. undertakes that all data and information used in this GRAS determination for use of the yBBS002 yeast strain in brewing will be made available for the FDA to review and copy at the following address:

Berkeley Brewing Science, Inc.
Attention: Charles Denby
2332 4th Street Suite E
Berkeley, CA 94710
USA

Data will be sent to the FDA upon request.

1.6. Freedom of Information Act, 5 U.S.C. 552

No data or information contained in parts 2 through 7 of this GRAS notice are exempt from disclosure under the Freedom of Information Act, 5 U.S.C. 552.

To the best of our knowledge, this GRAS notice for Saccharomyces cerevisiae yBBS002 and its use in brewing is a complete, representative, and balanced submission that includes both favorable and unfavorable information, known to Berkeley Brewing Science, Inc. and pertinent to the evaluation of the safety and GRAS status of the use of the substance.

(b) (6)

6/19/18

Charles Denby, Ph.D. Date
Chief Executive Officer
Berkeley Brewing Science, Inc

PART 2: IDENTITY, METHOD OF MANUFACTURE, SPECIFICATION, AND PHYSICAL OR TECHNICAL EFFECT

2.1. IDENTITY OF THE NOTIFIED SUBSTANCE

The subject of this notification is an industrial brewing *Saccharomyces cerevisiae* strain called yBBS002. This strain carries four recombinant genes encoding HMG-CoA reductase and FPP synthase derived from *Saccharomyces cerevisiae* (baker's and brewing yeast), a linalool synthase derived from *Mentha citrata* (mint), and a geraniol synthase derived from *Ocimum basilicum* (basil).

2.2 HOST MICROORGANISM

The host yeast strain from which yBBS002 was derived is an industrial brewing strain of *Saccharomyces cerevisiae* called California Ale Yeast (CAY), which has been commonly used in commercial beer production in the USA (for example, as in Sierra Nevada Pale Ale).

2.2.1. History of use

Saccharomyces cerevisiae has been used in food production for thousands of years. The first evidence of beer production comes from ancient Iran, Iraq, and Egypt, although spontaneous fermentations resulting in beer were likely happening far earlier than that, as wild yeasts in the air will ferment most grain left unattended. Ancient peoples utilized yeast in the production of other fermented foods and baked goods as well. Today yeast is an integral component in many industries, and millions of tons are produced commercially each year.

S. cerevisiae has also served as a model organism in scientific research. Many aspects of its biology makes *S. cerevisiae* attractive to study: it is an single-celled eukaryotic organism; it has a compact genome; many of its genes are homologous to human genes; it is genetically tractable, it has a short generation time, it is easily cultured, and it is known to be non-pathogenic and safe for industrial food processes. As the first eukaryotic genome to be completely sequenced, yeast have contributed to research on topics including DNA damage and repair, meiosis, recombination, gene function, cell cycle, and disease.

The host strain, California Ale yeast, is the workhorse strain of many breweries and homebrewers. It is extremely versatile and can be used in almost any style of ale and in other fermented beverages.

2.2.2. Taxonomy

The host strain, CAY, is an isolate from California in the USA and belongs to the group of brewing strains known as ale strains. Ale strains are classified as belonging to the *Saccharomyces cerevisiae* species. This designation relies on identification methods based on molecular analysis of the yeast genome.

2.2.3. Characteristics

The CAY strain is tetraploid and exhibits low sporulation efficiency. It is commonly used in industry as a liquid ale yeast. The production during manufacturing and reproduction during fermentation occurs asexually through budding.

CAY is a strong fermenter over a wide temperature range with high attenuation and high alcohol tolerance. It is recommended for use in production of almost any style of ale, ciders, and perry. It is noted for its clean flavors, balance, and extreme versatility.

2.3. DONOR ORGANISMS

2.3.1. Taxonomy

The following organisms contributed DNA to the modified organism yBBS002:

Saccharomyces cerevisiae

The history of use of this species is given in Section 2.2.1. The donor DNA sequences derive from the widely used *S. cerevisiae* lab strain, S288C. S288C was isolated through genetic crosses for biochemical studies by Robert Mortimer[3] and serves as the reference sequence strain in the Saccharomyces Genome Database. A majority of the gene pool of S288C is contributed by a strain that was isolated from rotting figs (88%); the remaining genetic material is contributed by baking yeasts and other yeast isolated from rotting fruit. In addition, the two sequences isolated from S288C are 99% identical to the sequences from the host strain, and brewing organism, CAY.

Mentha citrata

This plant species, also known as *Mentha x piperita* L. var. *citrata*, *Mentha aquatica* var. *citrata*, and commonly as bergamot mint, or yerba buena in Central America, is an herb belonging to the mint family. It has been used medicinally, in tea form, and as a cooking herb. Its strong odor is due to the essential oil containing almost entirely linalool and linalyl acetate[4]. The modified organism yBBS002, contains DNA that codes for the amino acid sequence of linalool synthase derived from *Mentha citrata*.

Ocimum basilicum

This plant species, known as basil, is also an herb in the mint family. There are many varieties of *Ocimum basilicum*, used widely throughout the world as a culinary ingredient. The essential oil contains many volatile chemical constituents, including geraniol; The modified organism yBBS002, contains DNA that codes for the amino acid sequence of geraniol synthase derived from *O. basilicum*.

2.3.2. Genetic material from donor organisms

The ScHMGR gene

The ScHMGR gene is derived from the HMG1 gene sequence present in *S. cerevisiae* strain S288C (chromosome XIII). HMG1 is one of two endogenous HMG-CoA reductase (HMGR) isozymes present in the yeast genome. The HMG1 gene codes for a multi-domain protein, composed of a membrane localization domain and a enzyme catalytic domain. The ScHMGR gene used in yBBS002 is composed of the enzymatic coding sequence only (i.e. lacks the membrane localization domain of HMG1). The impact of ScHMGR heterologous expression on yeast metabolism has been evaluated previously, and is discussed in Section 6.2.2. The entire sequence of the HMG1 gene from this strain has previously been described[5].

The ScHMGR gene was cloned after amplification by PCR of *S. cerevisiae* S288C genomic DNA. The gene was sequenced by Berkeley Brewing Science Inc. and the sequence in FASTA format is given in Appendix 1. After the gene was sequenced, all further cloning was done by Golden Gate Assembly, a restriction enzyme-based cloning method that avoids mutations introduced by PCR-based methods. The length of the amino acid sequence is 527 and the molecular weight of this protein has been determined to be 55,763 Da. The single-domain form is approximately half the length of the endogenous multi-domain protein.

The ScFPPS* gene

The ScFPPS* gene is derived from the ERG20 gene sequence present in *S. cerevisiae* strain S288C (chromosome X). The ERG20 gene encodes a farnesyl pyrophosphate (FPP) synthase, containing both dimethylallyltransferase and geranyltransferase activities. FPP synthase catalyzes the sequential condensation of two isopentyl pyrophosphate (IPP) molecules with dimethyl pyrophosphate (DMAPP) to form FPP, which serves as a precursor for essential metabolites including hemes and sterols. Geranyl pyrophosphate (GPP), the immediate precursor of linalool and geraniol, is formed as an intermediate of the sequential reactions. While the high processivity of the wild-type FPPS limits the intracellular availability of GPP, the ScFPPS* protein containing F96W and N127W mutations exhibits reduced processivity, thereby increasing production of GPP-derived end products[6]. The entire sequence of the ERG20 gene from strain S288C has previously been described[7].

The ScFPPS* gene used to construct the yBBS002 is derived from the gene variant containing the F96W and N127W mutations. The gene was recoded to avoid potential recombination with the native gene in the host organism, synthesized by oligonucleotide assembly, cloned, and sequenced by Berkeley Brewing Science Inc. After the gene was sequenced, all further cloning was done by Golden Gate Assembly, a restriction enzyme-based cloning method that avoids mutations introduced by PCR-based methods. The sequence in FASTA format is given in Appendix 2. The length of the amino acid sequence is 354 and the molecular weight of this protein has been determined to be 40,739 Da.

The McLIS gene

The McLIS gene is derived from *Mentha citrata* and encodes a monoterpene synthase gene that forms linalool from the precursor molecule geranyl pyrophosphate. The entire peptide sequence has been described previously[4]. In plants, monoterpenes are biosynthesized in chloroplasts; accordingly, plant monoterpene synthases typically contain an N-terminal plastid targeting

sequence (PTS) composed of 20-80 polar amino acids. Once the PTS-containing peptide enters the chloroplast, the PTS is cleaved to produce a mature, active protein. Since yeast lacks chloroplasts, and the cellular machinery required to process the PTS-containing peptide into its mature form, a gene sequence coding for the peptide sequence of the mature protein was used.

The gene coding for the mature protein was synthesized by oligonucleotide assembly, cloned, and sequenced by Berkeley Brewing Science Inc. After the gene was sequenced, all further cloning was done by Golden Gate Assembly, a restriction enzyme-based cloning method that avoids mutations introduced by PCR-based methods. The sequence in FASTA format is given in Appendix 3. The length of the amino acid sequence is 542 and the molecular weight of this protein has been determined to be 63,579 Da. The truncated form is otherwise identical to the full length protein.

The ObGES gene

The ObGES gene is derived from *Ocimum basilicum* and encodes a monoterpene synthase gene that forms geraniol from the precursor molecule geranyl pyrophosphate. The entire sequence has previously been described[8]. While the GES protein is also expressed in the chloroplast of *O. basilicum*, the full-length non-truncated version is functional in yeast. Therefore, we expressed the full length version of the GES gene.

The gene coding for full length GES was synthesized by oligonucleotide assembly, cloned, and sequenced by Berkeley Brewing Science Inc. After the gene was sequenced, all further cloning was done by Golden Gate Assembly, a restriction enzyme-based cloning method that avoids mutations introduced by PCR-based methods. The sequence in FASTA format is given in Appendix 4. The length of the amino acid sequence is 569 and the molecular weight of this protein has been determined to be 65,134 Da.

The promoter sequences

The promoter sequences (i.e. genetic elements immediately 5' of the heterologous genes) are derived from *S. cerevisiae* strain S288C. The CCW12 promoter drives tHMG, the TEF1 promoter drives ERG20*, the HTB2 promoter drives LIS, and the HHF2 promoter drives GES. These gene promoters were chosen because they are commonly used for heterologous gene expression in yeast, are well characterized, and thought to drive constitutive expression based on their controlling "housekeeping" cellular functions, and their measured expression patterns. In endogenous context, the CCW12 promoter controls expression of a cell wall protein. The TEF1 promoter controls expression a translational elongation factor. The HHF2 and HTB2 promoters control histone proteins. The promoters used in this study consist of approximately 700 bp fragments (700, 700, 699, 676) ending at the base pair immediately preceding the gene's ATG start codon. Restriction enzyme sites were removed from the TEF1 and HHF2 promoters (BsaI and BsmBI, respectively) for compatibility with the cloning strategy; the CCW12 and HTB2 promoters were cloned directly from S288C genomic DNA.

The terminator sequences

The terminator sequences (i.e. genetic elements immediately 3' of heterologous genes) were isolated from *S. cerevisiae* strain S288C. The ENO1 terminator follows tHMG, the SSA1 terminator follows ERG20*, the ADH1 terminator follows LIS, and the PGK1 terminator follows GES. The terminators allow for correct termination of the mRNA elongation during gene expression. The terminators used in this study consist of 225 bp fragments starting immediately after the stop codon of each gene.

The ADE2 sequence

DNA sequences from the ADE2 locus are derived from the ADE2 locus sequence present in *S. cerevisiae* strain S288C (chromosome XV). DNA sequences from the ADE2 locus were used to target the integration of the donor DNA material by homologous recombination. Fragments corresponding to the 5' and 3' ends of the ADE2 locus were used to flank the ends of the DNA to be integrated. The 5' fragment includes the ADE2 promoter (nucleotide -800 to nucleotide -1 relative to ATG start codon), the ADE2 gene (nucleotide +1 to nucleotide +1715), and the terminator (nucleotide +1716 to nucleotide +1831). The 3' fragment includes 770 nucleotides 3' of the terminator (nucleotide +1832 to nucleotide +2601). The ADE2 gene (systematic name YOR128C, SGD ID S000005654) encodes the phosphoribosylaminoimidazole carboxylase enzyme (EC 4.1.1.21) in the purine nucleotide biosynthesis pathway. In addition to targeting DNA to be integrated to the correct genomic site, incorporation of the ADE2 gene allows for selection of successfully transformed yeast by purine biosynthesis auxotrophy.

2.4. THE MODIFIED MICROORGANISM

2.4.1. Final construct used in the integration strategy

2.4.1.1. General construction strategy

In order to generate yBBS002, we developed a strategy that allows for stable incorporation of heterologous genetic material into tetraploid brewer's yeast without the use of bacterially-derived selection markers (Figure 2). The strategy uses two-steps: First, the ADE2 gene at the target integration locus is deleted in the parent brewer's yeast strain, resulting in a ADE2 deletion strain (ADE2Δ). Second, the genetic construct to be integrated in the final strain, which includes the ADE2 gene as well as the monoterpene biosynthesis pathway genes, is integrated into the ADE2Δ locus. The integration methodology uses Cas9 to generate double strand breaks at the target locus while supplying a repair template that contains homology to either side of the double-strand break. Because the parent brewer's yeast strain is tetraploid, achieving a successful integration requires that four copies of the target locus are created and repaired. This is achieved by supplying a plasmid containing Cas9, as well as a "repair template," which contains homology elements flanking the heterologous DNA. The target locus, ADE2, encodes a gene in the purine biosynthesis pathway, the deletion of which results in accumulation of an intermediate metabolite with red pigment. Therefore, successful deletion of the gene results in a red colony morphology when grown on appropriate medium. Conversely, in the second transformation step, the ADE2 gene is restored, and successful transformation

events are determined by selecting transformants with the white-colony morphology. A detailed account of the integration events used to create yBBS002 is described below:

2.4.1.2. Generating the ADE2Δ strain

ADE2 was deleted in host strain CAY by transforming a linear repair template containing 800 bp DNA fragments corresponding to regions immediately 5' and 3' of the ADE2 coding sequence together with a plasmid containing Cas9, a guide RNA targeting ADE2, and a G418 resistance marker. Positive transformants were selected by growth on YPD media supplemented with 200 mg/L G418. The resulting ADE2Δ strain, with all four ADE2 copies successfully deleted, was resistant to G418 supplementation and exhibited red colored morphology. Furthermore, because the strain could no longer biosynthesize purine DNA bases *de novo*, it exhibited adenine auxotrophy (i.e. could not be grown on media devoid of adenine). Following confirmation of the successful deletion, the ADE2Δ strain was cured of the cutting plasmid that contained the G418 resistance gene by several passages on YPD media. Plasmid loss was confirmed by testing for growth on G418 and confirming sensitivity (Figure 8).

2.4.1.3. Generating yBBS002 from the ADE2Δ strain

Once the ADE2Δ strain was obtained, a second transformation was performed to incorporate the monoterpene biosynthesis pathway genes (the integration cassette), thereby generating yBBS002. The resulting ADE2Δ strain was then transformed with the monoterpene pathway integration cassette, which contained homology to the ADE2Δ locus together with a second Cas9 plasmid. This plasmid contained a guide RNA targeting the ADE2 3' region and a G418 resistance marker, and transformants were selected by growth on YPD media supplemented with 200 mg/L G418. Because the monoterpene pathway cassette contains the ADE2 gene, successful transformants were also selected based on adenine prototrophy by growth on adenine-deficient media. Because the host organism has four allelic copies of the ADE2 deletion, stable integration requires repair at multiple ADE2Δ genomic loci. The integration event was also confirmed by diagnostic PCR (see Section 2.4.2.2). Following confirmation, the strain was re-streaked over several passages on YPD media until it no longer contained the Cas9 plasmid. Plasmid loss was confirmed by testing for growth on G418 and confirming sensitivity, resulting in a transformant that was the progenitor of strain yBBS002. A schematic of the method used is given in Figure 2.

The ADE2 deletion repair template was constructed by PCR using a high fidelity DNA polymerase (PrimeSTAR GXL DNA polymerase, Takara) and S288C genomic DNA as template. The ADE2 5' and 3' flanking sequences were subsequently cloned by Gibson Assembly into a linearized vector containing an *E. coli* origin and marker, giving plasmid pBS004. Figure 3 is a schematic of the ADE2 deletion repair template and the cassette as it is located within the resulting plasmid. The linear cassette is released from the plasmid after digestion with the restriction enzyme NotI. Digestion with NotI results in a blunt ended DNA fragment with 100% homology for the recombinogenic flanking sequences. A schematic of the linear ADE2 deletion repair template, showing the location of the blunt-end restriction enzyme NotI, is shown in Figure 3 and the sequence in FASTA format is given in Appendix 5.

The integration cassette parts were constructed by PCR using a high fidelity DNA polymerase (PrimeSTAR GXL DNA polymerase, Takara) and Gibson assembly using Gibson Assembly Master Mix (New England Biolabs). After sequence confirmation of each part plasmid (containing a promoter, coding sequence, or terminator), the "pathway" plasmid containing the full integration cassette was assembled by the standard Golden Gate method using type II restriction enzymes and T7 DNA ligase (New England Biolabs) (for addition detail, see schematized assembly strategy in Figure 5). Figure 6 is a schematic of the pathway cassette and the cassette as it is located within the resulting plasmid pCV265. The linear cassette is released from the plasmid after digestion with the restriction enzymes NotI and PstI. Digestion with NotI and PstI results in a blunt ended DNA fragment with 100% homology for the recombinogenic flanking sequences. A schematic of the linear pathway cassette, showing the location of the blunt-end restriction enzymes NotI and PstI, is shown in Figure 6 and the sequence in FASTA format is given in Appendix 6.

Cas9 plasmids containing the Cas9, a G418 marker, and a dropout GFP were constructed by by PCR using a high fidelity DNA polymerase (PrimeSTAR GXL DNA polymerase, Takara) and Gibson assembly using Gibson Assembly Master Mix (New England Biolabs). The Cas9 plasmids used in this study, containing a guide RNA in place of the GFP, were assembled by the standard Golden Gate method using type II restriction enzymes and T7 DNA ligase (New England Biolabs). A schematic of plasmid pCV166, targeting the ADE2 coding sequence and used to delete ADE2, is shown in Figure 4 and a schematic of plasmid pWL112, targeting the ADE2 3' region and used to insert the monoterpene pathway cassette, is shown in Figure 7.

2.4.1.4. Detailed description of the final construct

The final construct used for the transformation of the host organism, designated as the integration cassette, was isolated from plasmid pCV265 by digestion using the NotI and PstI restriction enzymes. The integration cassette consists of the "monoterpene pathway cassette," which is the fragment to be integrated, flanked by homologous sequences of the *S. cerevisiae* ADE2 locus and the ADE2 open reading frame. The monoterpene pathway cassette contains the *S. cerevisiae* ScHMGR gene cloned between the *S. cerevisiae* CCW12 promoter and the *S. cerevisiae* ENO1 terminator, the *S. cerevisiae* ScFPPS* gene cloned between the *S. cerevisiae* TEF1 promoter and the *S. cerevisiae* SSA1 terminator, the *M. citrata* McLIS gene cloned between the *S. cerevisiae* HTB2 promoter and the *S. cerevisiae* ADH1 terminator, and the *O. basilicum* ObGES gene cloned between the *S. cerevisiae* HHF2 promoter and the *S. cerevisiae* PGK1 terminator. Small synthetic linker scars (6-33 base pairs) used in the cloning strategy are also present in this cassette. In addition, all genes contain a two N-terminal amino acid linker (ScHMGR and ScFPPS* have Gly-Ser, McLIS and ObGES have Ile-Ser) used in the cloning strategy. A schematic representation of the integration cassette and its sequence in FASTA format are given in Figure 6 and Appendix 6.

2.4.2. The transformation events

2.4.2.1. Genetic material used for the transformation method

The Cas9 plasmid and the linear monoterpene pathway integration cassette excised from the vector pCV265 were the only DNA sequences used in the transformation method for strain yBBS002.

2.4.2.2. Screening method for transformants

The first transformation was completed using the Cas9 plasmid pCV166 and ADE2 deletion repair template and the cells were plated onto G418 containing media in order to select for transformed cells. A single colony with red colony morphology was chosen, streaked for purity and passaged for loss of pCV166 plasmid. The identity of the resulting ADE2Δ strain was confirmed by colony PCR. The ADE2Δ strain was then transformed with the Cas9 plasmid pWL112 and the monoterpene pathway integrating cassette and plated onto G418 containing media in order to select transformed cells. Colonies were then plated onto minimal media lacking adenine. Only successfully transformed cells were capable of forming colonies on such media and non-transformed cells did not grow. In addition, the transformed cells produced wild-type, white colonies. The integration event at all loci was confirmed by diagnostic PCR. Homozygosity at the integration locus was tested using primers targeted to the 5' and 3' junctions of desired allele and parent allele. The identity of the multi-gene integration was verified with primers targeted to each of the four promoter/gene junctions. Following confirmation, the strain was passaged on YPD media for pWL112 plasmid loss. Plasmid loss was confirmed by testing for growth on G418 and a colony that was sensitive to G418 was selected for further study (Figure 8). Functionality of the monoterpene pathway was then assessed by fermentation and monoterpene analysis using solid phase microextraction (SPME) coupled with gas chromatography mass spectrometry (Figure 9).

2.4.3. Genetic characterization of the modified microorganism

2.4.3.1. The loss of the Cas9 plasmid containing the antibiotic resistance gene

Loss of the plasmid containing the antibiotic resistance gene was confirmed by streaking yBBS002 onto G418-containing media and confirming sensitivity. Figure 8 clearly shows the yBBS002 is sensitive to this antibiotic like the parental strain CAY.

2.4.3.2. Genetic stability of the ADE2 allele

Since all four copies of the AE2 allele were replaced with four copies of the integration cassette, the stability of the cassette will not be affected by gene conversion. There is no reason to suspect that the stability of the integration cassette containing ADE2 allele would be any different than the native ADE2 allele.

2.4.4. Absence of difference between genetic profiles of the transformed and the host strain

The genome of *Saccharomyces cerevisiae* contains long terminal repeat sequences known as δ elements. These δ elements are the remnants of Ty1 transposon integration events. The number and location of these δ elements are specific to a strain and have been used to fingerprint and differentiate between strains of *S. cerevisiae*. Using PCR and primers 5'-

GTGGATTTTTATTCCAAC-3' and 5'-TCAACAATGGAATCCCAAC-3' (delta2/delta12 primer pair) to amplify these δ sequences we have verified the genetic relationship between the host strain and yBBS002 (Figure 10). This technique is commonly employed to differentiate between industrial *S. cerevisiae* strains.

We therefore conclude that the yBBS002 strain is not different from the CAY parent strain except for the monoterpene pathway cassette present at the ADE2 locus. No major DNA reorganization event has occurred upon integration of the monoterpene pathway cassette into the yBBS002 strain.

2.5. METHOD OF MANUFACTURE OF THE MODIFIED MICROORGANISM

Propagation of the yBBS002 yeast strain does not require use of selective media. The yBBS002 liquid brewing yeast can therefore be manufactured in the exact manner as all liquid yeast used in brewing or winemaking. A brief description of the manufacturing of yBBS002 liquid yeast is given in Figure 11.

2.6. LEVELS OF USE OF THE SUBSTANCE

We recommend that brewers use approximately 1 million cells per milliliter of wort per degree Plato for brewing wort fermentation, as is standard industrial practice. At this inoculation level, alcoholic fermentation will be efficiently conducted and hoppy aromas at the level of 0.1-0.5 mg/L will be produced (similar to those of a pale ale).

PART 3: DIETARY EXPOSURE

3.1. INTENDED USE OF yBBS002

Berkeley Brewing Science, Inc. has proposed the use of yBBS002 in brewing to impart hoppy flavors to beer during fermentation. This strain produces final concentrations of target flavor molecules in beer that are similar to those achieved with traditional hopping, without the use of hops. It is intended to be added at approximately 1 million cells per milliliter of wort per degree Plato for brewing wort fermentation, as is standard industrial practice.

3.2. ESTIMATED DIETARY EXPOSURE

3.2.1. History of consumption

Saccharomyces cerevisiae has been used in food production for thousands of years. The first evidence of beer production comes from ancient Iran, Iraq, and Egypt, although spontaneous fermentations resulting in beer were likely happening far earlier than that, as wild yeasts in the air will ferment most grain left unattended. Today yeast is an integral component in many industries, and millions of tons are produced commercially each year.

The monoterpenes linalool, geraniol and citronellol have been identified as primary flavor determinants by several sensory analyses of hop extract aroma[9 10 11] and finished beer taste and aroma[10 1 12 13]. Together, they are major drivers of the floral aroma of Cascade hops[1], the most widely-used hop in American craft brewing[14]. Commonly found in regularly-hopped beers, these monoterpenes are present at much higher concentrations in (currently popular) heavily dry-hopped beers.

3.2.2. Estimated consumption

Beer produced with yBBS002 will contain similar levels of linalool, geraniol, and citronellol as are present in traditionally hopped beer. All three of these compounds are GRAS flavoring agents which may be safely used in food in accordance with good manufacturing practice[15].

Exposure to the engineered yeast in the finished beer will be extremely limited. Yeast material is removed from beer during commercial production, either by flocculation, filtration, or centrifugation. The least stringent method of purifying yeast from finished beer is flocculation (induced by cooling). Even using this method, only trace levels of yBBS002 yeast remain in finished beer. These levels are not detectable by optical density measurements, indicating that final concentrations are negligible. In addition, yBBS002 will not proliferate in packaged beer, but instead will decline over the shelf-life of the product.

yBBS002 produces concentrations of monoterpene flavor molecules that are similar to those achieved with traditional hopping. Consumption of these molecules in beer produced with yBBS002 should be equivalent to the consumption of beer made with traditional hopping.

PART 4 - SELF-LIMITING LEVELS OF USE

This part does not apply.

PART 5: EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

This GRAS conclusion is based on scientific procedures.

PART 6: NARRATIVE

6.1. SAFETY ASSESSMENT OF THE HOST STRAIN

The host yeast strain is an industrial brewing strain of *Saccharomyces cerevisiae* called California Ale Yeast (CAY) that has been commonly used in commercial beer production in the US.

S. cerevisiae is an organism that has an extensive history of safe use. It has been used for millennia in fermentation processes, such as bread leavening and beer and wine production. It is responsible for spontaneous fermentation of grains and grape juice[16].

S. cerevisiae is considered GRAS through its use in the brewing, baking, and winemaking industries. Its genome has been sequenced, and it has been determined that the yeast is free of known pathogenicity traits. Genetically modified yeast strains such as Lesaffre's malolactic yeast strain ML01 (GRN No. 120), Phyterra's urea degrading yeast strain ECMo01 (GRN No. 175), and Phyterra's low hydrogen sulfide yeast strain P1Y0 (GRN No. 350) have all been previously granted GRAS status by the US FDA.

In the 27th report of The Scientific Committee for Human Food of the European Community, the authors state that *Saccharomyces cerevisiae* has a safe history of use in food and belongs to a species that is known not to produce toxins. In addition, the Environmental Protection Agency (EPA) has included *S. cerevisiae* as a recipient microorganism for exemptions from EPA review and expedited EPA review (40 CFR 725.420). This exemption was made because this species is found to have little adverse effects. They also determined that the introduction of genetic material would not increase the potential for adverse effects, provided that the genetic material is limited in size, well characterized, free of certain sequences, and poorly mobilizable.

The yBBS002 yeast strain belongs to the *Saccharomyces cerevisiae* species which have been used for more than 7,000 years by humans in fermented beverages. Given its history of use, it can therefore be concluded that this species will remain GRAS, even after genetic transformation, as long as no harmful DNA material is added to it.

6.2. SAFETY ASSESSMENT OF THE GENETIC MATERIAL USED TO CONSTRUCT THE MODIFIED ORGANISM

6.2.1. Foreign genetic material source and product

6.2.1.1. The McLIS gene

Source: *Mentha citrata*

The McLIS gene codes for the mature form of *Mentha citrata* linalool synthase as described in Section 2.3.2. *M. citrata* is an edible plant species that is used in traditional medicines, foods, and cosmetic. Given that mint essential oil is composed of 60-70% linalool and derivatives thereof[4, 17], mint leaves contain this protein at appreciable levels, which have a long history of safe human consumption.

Given its history of use, this plant raises no consumer safety concerns and can reasonably be submitted as safe.

Product: Linalool synthase

The amino acid sequence coded for by McLIS is identical to the *Mentha citrata* sequence, with the additional N-terminal dipeptide (Ile-Ser) which confers stability in proteins that are heterologously expressed in yeast. The fact that the yBBS002 yeast produces linalool provides further support that the linalool synthase functions as in mint plant. This demonstrates that the secondary and tertiary structures of the recombinant protein have not suffered major changes compared to the native linalool synthase.

Hence, expressing the McLIS gene in *Saccharomyces cerevisiae* has no major consequence on the properties of the linalool synthase, which can therefore be assessed as safe as the native linalool synthase in *Mentha citrata*.

6.2.1.2. The ObGES gene

Source: *Ocimum basilicum*

The ObGES gene was derived from a yeast codon-optimized version of the *O. basilicum* gene as described in Section 2.3.2. *Ocimum basilicum* is an edible plant species that is commonly used in foods from many parts of the world.

Given its history of use, this plant raises no consumer safety concerns and can reasonably be submitted as safe.

Product: Geraniol synthase

The nucleotide sequence codes for the amino acid sequence of the native *Ocimum basilicum* peptide sequence, with the additional N-terminal stabilizing dipeptide (Ile-Ser). The fact that the yBBS002 yeast produces geraniol shows that the geraniol synthase is functional. This demonstrates that the secondary and tertiary structures of the recombinant protein have not suffered major changes compared to the native geraniol synthase.

Hence, expressing the ObGES gene in *Saccharomyces cerevisiae* has no major consequence on the properties of the geraniol synthase, which can therefore be assessed as safe as the native geraniol synthase in *Ocimum basilicum*.

6.2.2. Native genetic material source and product

6.2.2.1. The ScHMGR gene

Source: *Saccharomyces cerevisiae*

The ScHMGR gene was isolated from S288C as described in Section 2.3.2. The safety assessment of *Saccharomyces cerevisiae* has already been established in Section 6.1.

Product: HMG-CoA reductase

ScHMGR converts HMG-CoA into mevalonate, which is an early step in the ergosterol biosynthesis pathway. Ergosterol is a major component of yeast membranes, with total levels amounting to 1% of yeast cell dry weight on average. It is essential for normal membrane function, affecting membrane fluidity and permeability[18]. While modified ScHMGR activity likely affects levels of ergosterol pathway intermediates in yBBS002, previous data show that excess flux through the ergosterol pathway accumulates as squalene, a molecule that is abundant in olive oil and is present at high levels in a Mediterranean diet[19].

Heterologous expression of ScHMGR from the ADH1 promoter was shown to result in altered ergosterol biosynthesis pathway flux. While the levels ergosterol itself were not affected by this modification, the concentration of pathway intermediates was altered. In particular, squalene concentrations increased substantially—to ~1% of total DCW—while other pathway intermediates exhibited more modest changes (~2-fold). In yBBS002, ScHMGR is driven by a weaker promoter than pADH1 and squalene is therefore not likely to accumulate to such high levels. On the other hand, even if the modification did result in similarly high squalene levels, a person would have to consume thousands of liters of normally processed beer (or tens of liters of non-flocculated beer) in order to consume levels comparable to that of a heavy olive oil diet (i.e. 400 mg daily in a Mediterranean diet[20]).

The nucleotide sequence of the integrated S288C gene differs at only four nucleotides from the CAY gene, and these nucleotide differences do not correspond to a change in amino acids. We can also infer that the addition of the N-terminal amino acid linker Gly-Ser used in the cloning strategy does not affect the overall protein structure since the ScHMGR is functional.

Therefore, to the best of our knowledge, the ScHMGR gene product of the yBBS002 strain is similar to that of the parent CAY *S. cerevisiae* strain and does not raise any safety concern.

6.2.2.2. The ScFPPS* gene

Source: *Saccharomyces cerevisiae*

The ScFPPS* gene was isolated from a codon-optimized version of the gene as described in Section 2.3.2. The safety assessment of *Saccharomyces cerevisiae* has already been established in Section 6.1.

Product: FPP synthase

The wild-type FPP synthase produces both GPP and FPP; GPP, however, is mostly prevented from release into the cytosol as it serves only as an intermediate in FPP production. The ScFPPS* protein containing the F96W and N127W mutations exhibits reduced affinity for GPP but a slightly higher K_M for DMAPP compared to the wild-type, thereby releasing some amount of GPP and allowing for production of GPP-derived end products[6]. The two FPPS proteins, therefore, make the same products but in different ratios, and are the same size and structure.

While the nucleotide sequence has been codon optimized to avoid homologous recombination with the native copy, the amino acid sequence is identical to the S288C sequence, with the additional N-terminal two amino acid linker Gly-Ser used in the cloning strategy. We can infer, however, that the addition of the N-terminal amino acid linker does not affect the overall protein structure since the ScFPPS* is functional.

Therefore, to the best of our knowledge, the ScFPPS* gene product of the yBBS002 strain is similar to that of the parent CAY *S. cerevisiae* strain and does not raise any safety concern.

6.2.2.3 The promoter and terminator sequences

The promoter and terminator sequences were isolated from *S. cerevisiae* strain S288C as described in Section 2.3.2. The safety assessment of *Saccharomyces cerevisiae* has already been established in Section 6.1 and since the regulatory sequences do not code for any proteins, no further safety assessment needs to be performed.

The source of this material does not raise any safety concern.

6.2.2.4 The ADE2 sequence

Source: *Saccharomyces cerevisiae*

The ADE2 sequence (5'-ORF-3') was isolated from *S. cerevisiae* strain S288C as described in Section 2.3.2. The safety assessment of *Saccharomyces cerevisiae* has already been established in Section 6.1.

Product: Phosphoribosylaminoimidazole carboxylase

The ADE2 sequence is present in both host and donor strain; following its deletion it is subsequently re-inserted into the host strain. This was done to facilitate integration of the monoterpene biosynthesis pathway. The insertion reconstitutes the original locus. The nucleotide sequence of the S288C gene differs at only six nucleotides from the CAY gene. One of these differences corresponds to a change in amino acid. Nevertheless we can infer that these changes do not affect the overall protein structure since the ADE2 gene is functional.

The source of this material does not raise any safety concern.

6.2.3. Construction of the modified organism

6.2.3.1. Vectors used as source for the integration events

The ADE2 deletion repair template was constructed by PCR using a high fidelity DNA polymerase (PrimeSTAR GXL DNA polymerase, Takara). Plasmid pBS004 was obtained by subcloning the PCR fragments by Gibson Assembly into the linearized vector backbone. The linear cassette is released from the plasmid after digestion with the restriction enzyme NotI (Figure 3). Digestion results in a blunt ended DNA fragment with 100% homology for the recombinogenic flanking sequences.

The pathway integration sequence was constructed by the standard Golden Gate method. Plasmids containing each of the four monoterpene pathway genes (plus promoter and terminator) were assembled into a vector containing the ADE2 flanking sequences and open reading frame, resulting in plasmid pCV265 (Figure 5). The linear cassette is released from the plasmid after digestion with the restriction enzymes NotI and PstI (Figure 6). Digestion results in a blunt ended DNA fragment with 100% homology for the recombinogenic flanking sequences.

6.2.3.2. Transformation of the host strain with the deletion repair template

The Cas9 plasmid and the linear ADE2 deletion repair template excised from the vector pCV166 were the only DNA sequences used in the transformation method.

The CAY strain was transformed using the Cas9 plasmid pCV166 (targeting the ADE2 coding sequence) and ADE2 deletion repair template and plated onto G418 containing media in order to select for transformed cells. A single colony with red colored morphology was chosen and streaked for purity. Identity of the resulting ADE2Δ strain was confirmed by colony PCR.

6.2.3.3. Transformation of the host strain with the integration cassette

The Cas9 plasmid and the linear monoterpene pathway integration cassette excised from the vector pCV265 were the only DNA sequences used in the transformation method.

The ADE2Δ strain was transformed with the Cas9 plasmid pWL112 (targeting the ADE2 3' region) and the monoterpene pathway integration cassette and plated onto G418 containing media in order to select transformed cells. Colonies were then plated onto minimal media lacking adenine. Only successfully transformed cells were capable of forming colonies on such media and non-transformed cells did not grow. In addition, the transformed cells exhibited white colored morphology. The integration event at all loci was confirmed by diagnostic PCR. Homozygosity at the integration locus was tested using primers targeted to the 5' and 3' junctions of desired allele and parent allele. The identity of the multi-gene integration was verified with primers targeted to each of the four promoter/gene junctions. Following confirmation, the strain was re-streaked over several passages on YPD media until it no longer contained the Cas9 plasmid. Plasmid loss was confirmed by testing for growth on G418 and a colony that was sensitive to G418 was selected for further study. Functionality of the monoterpene pathway was then assessed by fermentation and monoterpene analysis using solid phase microextraction (SPME) coupled with gas chromatography mass spectrometry (Figure 9). A schematic of the transformation method used is given in Figure 2.

Standard microbiological methods were employed in order to verify that the Cas9 plasmid containing the G418 antibiotic marker had been eliminated from the yBBS002 strain. These methods relied on the fact that the yBBS002 strain had lost its ability to grow on G418 containing media, indicating the absence of the plasmid (Figure 8).

The results show that the Cas9 plasmid containing the G418 marker is not present in the yBBS002 strain.

6.3. SAFETY ASSESSMENT OF THE MODIFIED ORGANISM

6.3.1. Characterization of the transformation event

The genome of *S. cerevisiae* contains long terminal repeat sequences known as δ elements. These δ elements are the remnants of Ty1 transposon integration events. The number and location of these δ elements are specific to a strain and have been used to fingerprint and differentiate between strains of *S. cerevisiae*. Using PCR and primers 5'-GTGGATTTTTATTCCAAC-3' and 5'-TCAACAATGGAATCCCAAC-3' (delta2/delta12 primer pair) to amplify these δ sequences we have verified the genetic relationship between the host strain and yBBS002 (Figure 10).

Loss of the plasmid containing the antibiotic resistance gene was confirmed by streaking yBBS002 onto G418-containing media and confirming sensitivity. Figure 8 clearly shows the yBBS002 is sensitive to this antibiotic like the parental strain CAY.

The yBBS002 strain has been genetically characterized. No antibiotic resistance sequence is present in the genetic material of strain yBBS002. Moreover, the CAY and yBBS002 strains have identical transposon fingerprinting patterns, providing evidence that no genomic rearrangement occurred during the transformation events. Therefore, to the best of our knowledge, it can be concluded that the yBBS002 strain is genetically substantially equivalent to the CAY host strain, except for the monoterpene pathway cassette present at the ADE2 locus.

6.3.2. Consequence of the genetic modification on the physiology of the yBBS002 strain

In total, four enzyme-encoding genes were integrated into the genome of yBBS002 as a single cassette. A detailed description of each enzyme activity is described below, and an overview of the relevant biosynthetic pathway is represented in Figure 1.

6.3.2.1 Overview of the function of the ScHMGR gene

The ScHMR gene codes for the catalytic domain of HMG-CoA reductase which converts HMG-CoA into mevalonate and is an essential enzymatic step in ergosterol and heme biosynthesis. This enzyme is 527 amino acids long and 55,763 Da in weight.

6.3.2.2 Overview of the function of the ScFPPS* gene

The ScFPPS* gene codes for a GPP/FPP synthase, which catalyzes the condensation of one or two isopentyl pyrophosphate (IPP) with dimethyl pyrophosphate (DMAPP) to form GPP or FPP. The endogenous enzyme is an essential enzymatic step in ergosterol and heme biosynthesis and generates FPP as the primary product. The ScFPPS* variant produces both FPP and GPP[6]. This enzyme is 354 amino acids long and 40,739 Da in weight.

6.3.2.3. Overview of the function of the McLIS gene

The McLIS gene codes for a linalool synthase, which converts GPP to linalool. This enzyme is expressed endogenously in mint plant (*Mentha citrata*). It is 542 amino acids long and 63,579 Da in weight.

6.3.2.4 Overview of the function of the ObGES gene

The ObGES gene codes for a geraniol synthase, which converts GPP to geraniol. This enzyme is expressed endogenously in basil plant (*Ocimum basilicum*). It is 569 amino acids long and 65,134 Da in weight.

6.3.2.5. Manufacture of liquid yeast during fed-batch aerobic cultures

Section 2.5 gave an overview of the method of manufacture of the yBBS002 strain in the form of liquid yeast. This method of manufacture is identical to that used for the current commercial manufacture of liquid brewing yeast obtained with the host strain CAY. No difference should be observed between CAY and yBBS002 during the manufacturing process.

6.3.2.6 Growth and fermentation rates of yBBS002 and CAY strains during laboratory-scale fermentations

Brewing trials performed in the laboratory have shown that the yBBS002 strain and the CAY strain have similar fermentation rates. The results have also been confirmed by larger scale fermentation trials (Figure 13). No growth advantage was found for the yBBS002 strain, which is virtually identical to the parent strain CAY. Therefore, the presence of the monoterpene pathway cassette does not give the yBBS002 strain any growth advantage and strain yBBS002 can be regarded as substantially equivalent to the wild type strain CAY, with an additional capacity for monoterpene production. All data indicate that the only substantial difference between the yBBS002 strain and the CAY strain is the capacity of the yBBS002 strain to produce linalool, geraniol, and citronellol.

Therefore it can be concluded that the yBBS002 strain is substantially equivalent to the host strain CAY except for the capacity of the yBBS002 strain to produce linalool, geraniol, and citronellol.

6.3.2.7 Sensory analysis of beer brewed with yBBS002

In order to compare the flavors conferred by the traditional brewing process with the flavors conferred by yBBS002, fermentations were performed with the parent strain (with and without dry-hop additions) as well as with yBBS002 without dry-hop additions. Brewing Innovation Manager at Lagunitas Brewing Company (Petaluma, CA), Bryan Donaldson, performed sensory analysis on the beer samples. A panel of 27 double-blinded trained tasters determined that the finished beer fermented with yBBS002 was perceived to contain more hop flavor than the controls and exhibited a range of hop flavor/aroma intensity. Tasters noted that yBBS002 produced a variety of distinct product flavors including "nice floral and orange blossom notes" without any detectable off flavors. Given that the flavors associated with linalool, geraniol, and citronellol, (which impart flavors of sweet/floral, rose/floral, and citrus, respectively), these tasting notes are consistent with our findings that the only alteration to yeast metabolism are related to these monoterpenes. Further detail on methodology and results of sensory analysis experiments can be found in the supporting data.

6.3.2.8 Metabolomic comparison of yBBS002, CAY, and BSA

In order to evaluate whether of off-target metabolic alterations were introduced by the genetic modification, un-targeted metabolomics was performed on beer produced with yBBS002 and its parent strain, CAY. A method was used to measure the relative concentrations of myriad metabolites in beer produced with yBBS002, CAY, as well as another commonly used industrial brewing strain termed Belgian Saison Ale (BSA). Performing metabolite analysis on the BSA strain served as a point of reference for metabolic differences between *Saccharomyces cerevisiae* strains used in industrial brewing. In total, 1,315 distinct metabolites were measured using liquid chromatography-high resolution mass spectrometry. For each strain, four biological replicates were used to start 25 mL fermentations, which were sampled after five days of fermentation. In order to evaluate the extent to which the genetic modification affected metabolism, the relative levels of 1,315 compounds were measured, and only a single compound exhibited >10-fold difference between yBBS002 and parent strain CAY. The single compound was identified with targeted MS/MS as asparaginylglutamine (predicted by MSMS spectral deconvolution). This compound is a dipeptide, and is likely derived from protein degradation/metabolism. It is not a known toxin. Indeed, dipeptides are commonly observed by LCHRMS, and this species represents one of many molecules of this type. In contrast, when comparing the parent strain CAY to the other industrial brewing strain, BSA, 238 compounds exhibited variation by >10-fold. In other words, the novel yeast strain yBBS002 appears substantially equivalent to the parent strain CAY apart from the targeted introduction of linalool and geraniol biosynthesis.

6.3.3. Allergenic/Toxigenic potential of the monoterpene pathway enzymes

The ingestion of an enzyme is generally not considered a concern for food allergy. This is based on the following considerations:

1. Enzymes have a long history of safe use in food, with no indication of adverse effects or reactions.

2. The majority of proteins are not food allergens. A wide variety of enzyme classes and structures are naturally present in plant and animal based foods.

3. Enzymes in foods are typically present in very low concentrations. In addition, whole enzymes are rarely found present in beer (typically hydrolysis products only), and even full length, but denatured, protein has been shown to be very susceptible to digestion in the gastrointestinal system.

In order to further evaluate the possibility that the enzymes expressed from the integration cassette will cross-react with known allergens and induce a reaction in an already sensitized individual, a sequence homology to known allergens was assessed. Following the guidelines developed by FAO/WHO, 2001[21, 22] and modified by Codex Alimentarius Commission, 2009[23], the four enzymes were compared to allergens from the Food Allergy Research and Resource Program (FARRP) allergen protein database (http://allergenonline.org).

Alignment of the expressed proteins to each of the allergens and identity of hits with more than 35% identity over the full length of the alignment was analyzed. No significant homology was found between any of the expressed proteins and any of the allergens from the database mentioned above. More than 35% identity in the amino acid sequence of the expressed proteins using a window of 80 amino acids and a suitable gap penalty showed no matches to correctly annotated proteins. In addition, a search for 100% identity over 8 contiguous amino acids was completed; no significant homology was found for any of the expressed proteins.

On the basis of the available evidence, it is concluded that oral intake of the proteins expressed from the integration cassette in strain yBBS002 is not anticipated to pose any food allergenic or toxigenic concerns.

6.3.4. Presence of unintended gene products as a result of the transformation event

S. cerevisiae has been employed by humans in production of food and beverages for millenia. It is known that during this period, major genetic changes have occurred[24], however, fermentation products of this yeast have never been described as having adverse effects on humans (when consumed in quantities that are recommended for alcohol intake).

In the monoterpene pathway integration cassette of the yBBS002 strain, the ScHMGR, ScFPPS*, McLIS, ObGES genes and the promoter and terminator sequences are present in a different genetic environment than their homologous native sequences. Therefore, we tested whether the integration cassette contained open reading frames (ORFs) beyond the four that were intentionally introduced (Figure 12). ORFs were identified using the NCBI ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/), which searched for ORFs of more than 100 codons. The nucleotide sequence of the putative ORFs and of the deduced amino acid sequences are given in a FASTA format in Appendix 7. We found two ORFs originating from an *S. cerevisiae* parental sequence (inside ScHMGR or the CCW12 promoter, in the reverse sequence orientation), and two originating from the McLIS gene (in the reverse sequence orientation). These unintended ORFs do not raise safety concerns because 1) the chance that a randomly generated ORF will be translated into a stable peptide is low, and 2) in the unlikely event that either of the ORFs did form a stable peptide, the peptide sequences are unlikely to be allergenic or toxicogenic based on their lack of similarity with known allergens/toxins (http://allergenonline.org).

There are a number of requirements for an ORF to give rise to a gene product beyond a coding sequence. It must contain the regulatory information for substantial transcription (conserved genes contain promoter elements that drive transcription), the transcript must be resistant to rapid degradation (genes with appreciable expression contain 3' untranslated region that facilitate stability), the transcript must be efficiently translated (which depends on 5' sequence with a translation initiation site as well as the composition of codons), the peptide must be resistant to rapid degradation.

In the event that any of the putative ORFs are translated into a stable proteins, the amino acid sequences of the putative ORFs do not exhibit homology to known allergic or toxic proteins. Following the guidelines developed by the FAO/WHO[21, 22] and modified by the Codex

Alimentarius Commission[23], we compared the putative peptide sequences to known allergenic/toxic peptides and found no similarity.

In conclusion, given:

- that the peptide sequences of the putative ORFs are unlikely to be allergenic or toxicogenic based on their lack of similarity with known allergens/toxins,
- the very weak chances of the putative ORFs being expressed as a stable peptide, and
- the fact that putative ORFs 1 and 2 are fragments of an *S. cerevisiae* parental sequence,

There is no reason to believe that the yBBS002 strain will release toxic or allergenic substances as a result of the presence of unintended gene products.

6.4. SAFETY ASSESSMENT OF THE PRODUCT DERIVED FROM THE MODIFIED ORGANISM

6.4.1. Changes in brewing procedures as a consequence of the yBBS002 fermentation

The yBBS002 strain enables the brewer to to impart hoppy flavors to beer during fermentation. Figure 14 indicates changes occurring in brewing procedures as a consequence of the use of the yBBS002 yeast strain. The use of the yBBS002 yeast strain leads to no drastic changes in the brewing procedures. Only at the stage of dry hopping does the procedure vary. Consequently, the brewing procedure will be shorter.

6.4.2. Changes in beer composition as a consequence of the yBBS002 fermentation

6.4.2.1. Global characteristics of beer

Brewing trials indicated that several parameters measured in beer (such as degrees Plato) stay similar in beers produced from the yBBS002 strain and the CAY parent strain (Figure 13). This implies that the yBBS002 beer yeast does not change the global constitution of beer.

6.4.2.2. Flavor modification

Laboratory and pilot scale brewing trials showed that the use of yBBS002 beer yeast strain does not lead to any so-called off-flavors. Descriptive characters of the yBBS002 beer did not significantly vary from the control beers with dry hops.

6.4.2.3. Yeast cells and release of yeast products during brewing

After alcoholic fermentation, the viable yeast population starts to decrease in beer and yeast autolysis occurs. While many of the solid particles of the beer as well as the yeast have settled to the bottom of the fermented by this point, the majority of beers are also clarified by one or more of three processes: cooling (which leads to accelerated flocculation), centrifugation, and filtration.

Beer clarification is often performed by letting the solid particles of the beer sediment by gravity, followed by elimination of the sediments. This process is initiated by a "cold crash"--a drop in the temperature of the beer to 33-40 degrees Fahrenheit. Clarification depends on the flocculation ability of the yeast strain: a strong flocculator will result in clear beer, a weak flocculator will result in turbid beer. Both the parent strain CAY and yBBS002 efficiently flocculate, thus the majority of the yeast is purified from the resulting finished beer.

Native and heterologous proteins may be liberated if cells are allowed to lyse. However, clarification processes will remove the bulk of the proteins, as well as larger polypeptide fragments. Hence, only hydrolysis products such as smaller polypeptides and amino acids of these proteins will remain in the beer. The cellular content of an autolyzing yeast is rich in nucleases and phosphatases. As a result of yeast autolysis, nucleic material will normally be found in beer as single nucleic bases or small nucleic base chains.

It should also be noted that products of both the ScHMGR and the ScFPPS* proteins are likely to be found in beer as a result of fermentation by unmodified yeast. Therefore, the only difference that may be present between a CAY beer and a yBBS002 beer is the presence of proteolysis products of McLIS and ObGES, likely a minimal difference.

Our data show that the only significant difference between the CAY and the yBBS002 yeast is the presence of the integration cassette, leading to the presence of the HMG-CoA reductase, GPP synthase, linalool synthase, and geraniol synthase enzymes which allow linalool, geraniol, and citronellol production by yeast. Brewing trials showed that yBBS002 beers were not different in their global composition than CAY beers.

Depending on the clarification process, different proportions of yBBS002 yeast cells might be found in the final product. Since the donor DNA integrated into yBBS002 was derived from edible organisms, proteolysis products derived from the integration cassette would be ingested under normal circumstances; therefore the presence of a small proportion of yBBS002 cells in the final product - resulting in the presence of small polypeptides and amino acids - should not pose a safety risk.

6.4.2.4. Formation of unwanted substances in beer

The ScHMGR, ScFPPS*, McLIS, and ObGES genes present in the yBBS002 strain as the result of the genetic modification do not code for either toxic or allergenic proteins, nor proteins implicated in the formation of undesirable compounds.

We have carried out risk assessments related to the presence of potential unintended gene products as the result of the genetic modification and found the following:

- Analysis using the NCBI ORF Finder shows that the integrated region contains 4 putative open reading frames;
- Our data suggests that they are not subsequently translated into proteins;
- Putative ORF 1 and 2 correspond to sequences present in *S. cerevisiae*. In the unlikely event that these ORFs are translated into proteins, they would be found

in both yBBS002 and the parental strain. As the parental strain has never been shown to release unwanted products corresponding to these sequences, neither should the yBBS002 strain.

These considerations lead us to conclude that allergenic or toxic risks related to the presence of these putative ORFs within the new yBBS002 strain are negligible. Moreover, to the best of our knowledge, the use of the yBBS002 strain in brewing will not lead to the release or the enhancement of the presence of undesirable compounds in beer.

PART 7: SUPPORTING DATA AND INFORMATION

Work underlying the development of yBBS002 has been published (*Nature Communications*, March 2018). See attached paper.

All information indicated in the list of Appendices and References is generally available.

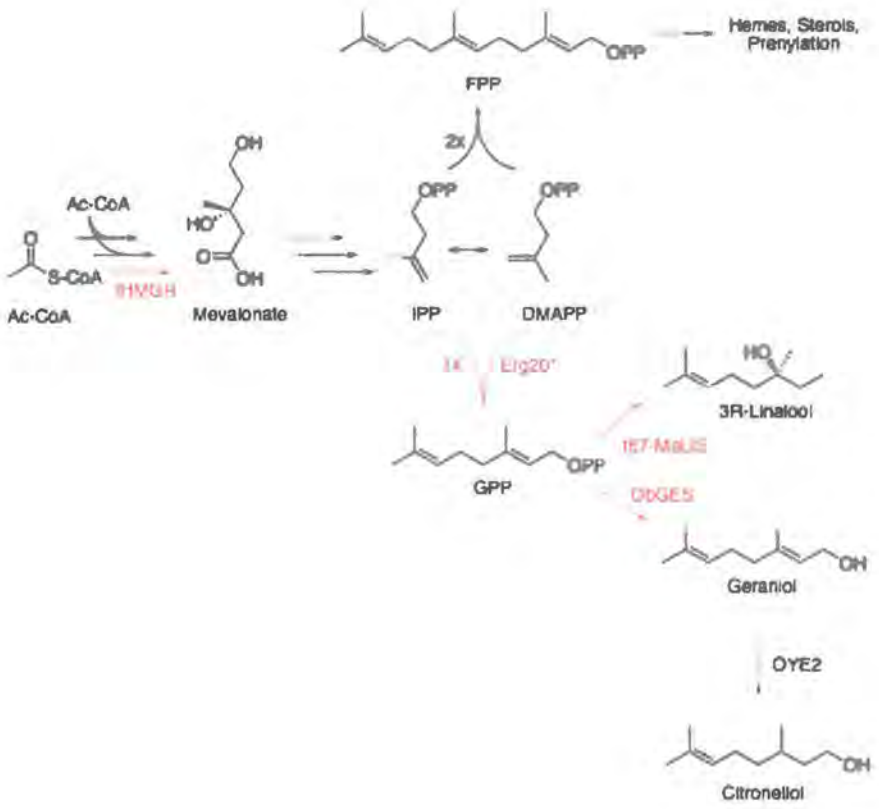

Figure 1: Monoterpene biosynthesis pathway in *Saccharomyces cerevisiae* harboring the monoterpene pathway integration cassette

Red arrows correspond to activities encoded by genes integrated into strain yBBS002.

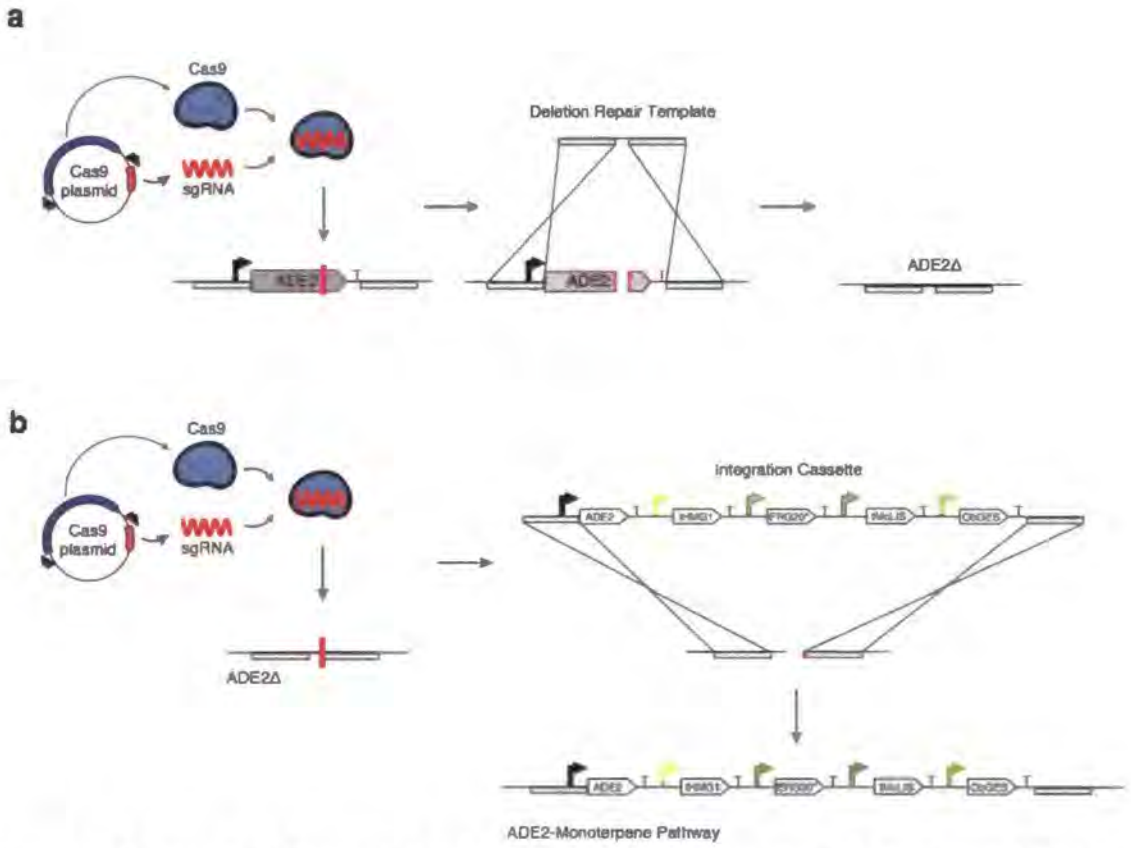

Figure 2: A schematic illustrating the strategy used to construct the commercial strain, yBBS002.

Note that a single copy of ADE2 is represented in the diagrams for simplicity; tetraploid brewer's yeast strains contain four copies of each gene in their genome. The host strain was co-transformed with a Cas9/sgRNA plasmid and repair template, which targeted a double-stranded break (DSB) in the ADE2 coding sequence and resulted in the deletion of the ADE2 gene. The ADE2Δ strain was then co-transformed with a second Cas9/sgRNA plasmid and integration cassette, which includes a copy of the ADE2 gene.

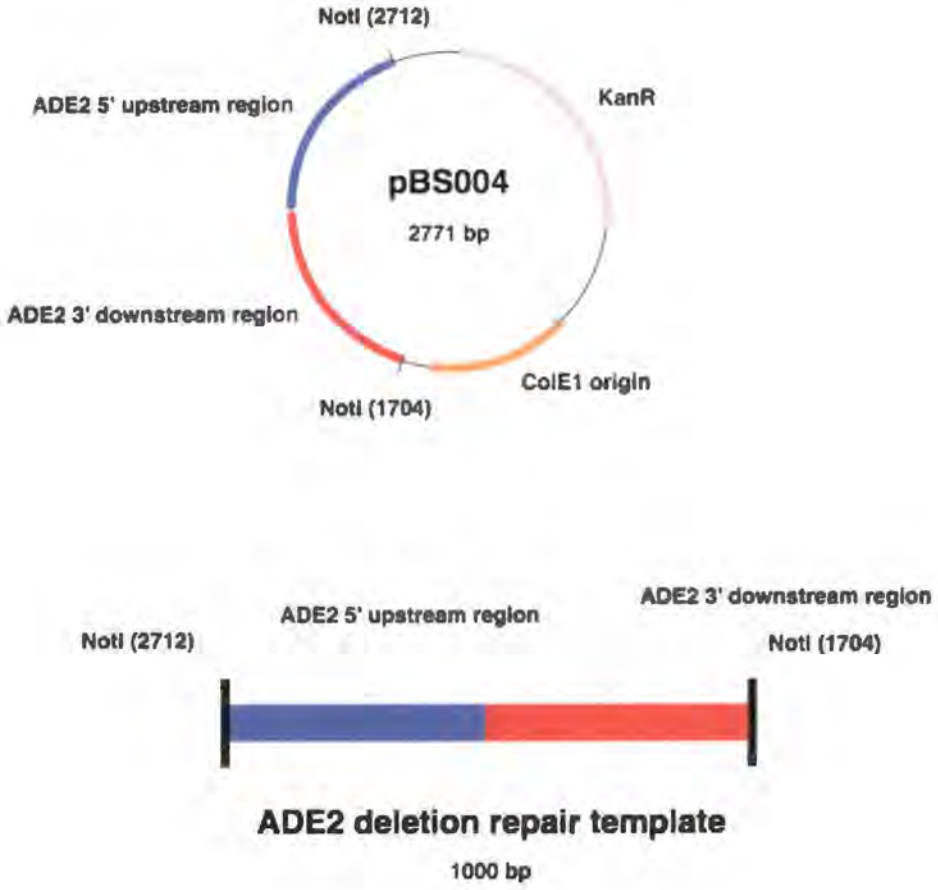

Figure 3: A schematic of the ADE2 deletion repair template within the plasmid vector pBS004 and the linear cassette without the vector backbone

Plasmid includes the following genetic elements: KanR encodes bacterial resistance to the antibiotic kanamycin, ColE1 is an origin of replication for propagation in *E. coli*, and the ADE2 5' and 3' regions are sequences immediately upstream and downstream of the yeast ADE2 coding sequence which facilitate homologous recombination in the host strain. NotI restriction sites are represented by solid black bars. The pBS004 plasmid is linearized before yeast transformation by digestion with NotI.

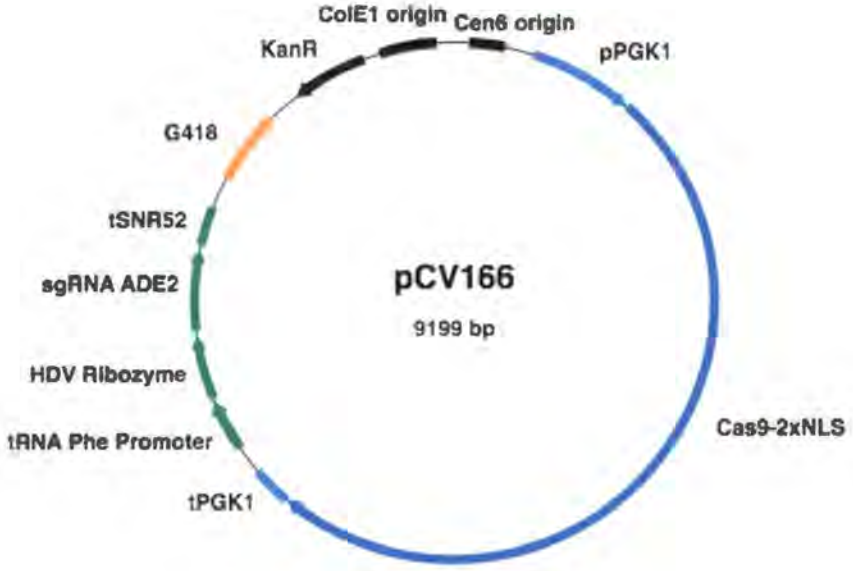

Figure 4: A schematic of the Cas9 plasmid pCV166 targeting the ADE2 coding sequence

pCV166 includes the following genetic elements: Cas9-2xNLS encodes a Cas9 protein with a nuclear localization tag, flanked by regulatory elements (PGK1 promoter and terminator), and the tRNA promoter, HDV ribozyme, and SNR52 terminator regulate expression of the guide RNA targeting ADE2. Additionally, it contains an origin of replication (ColE1 origin) and the KanR drug resistance marker for propagation and selection in *E. coli*, and an origin of replication (Cen6 origin) and the G418 drug resistance marker for propagation and selection in yeast.

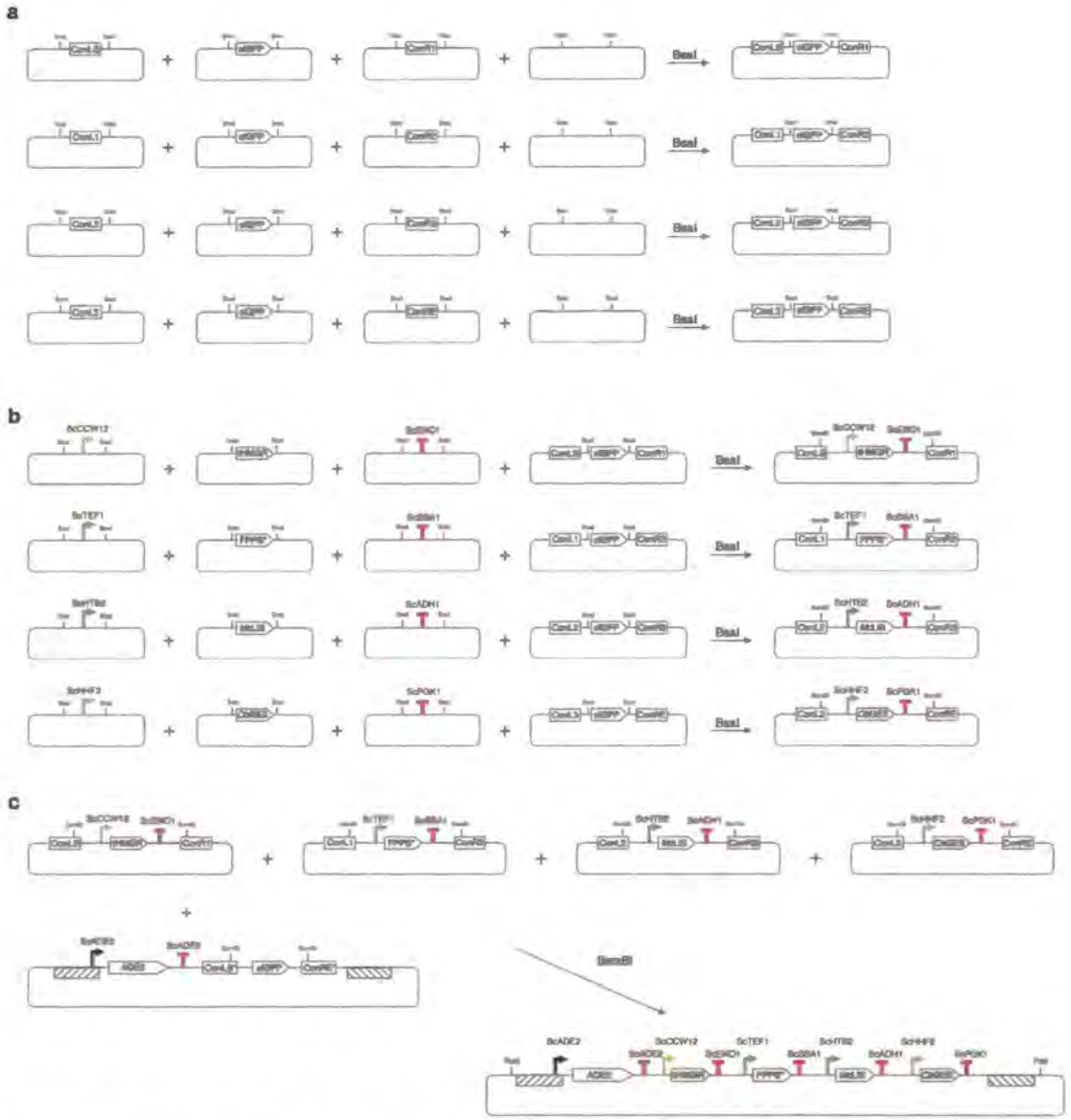

Figure 5: A schematic illustrating the construction of plasmid pCV265

(**a**) Assembly of backbone plasmids to be used in cassette plasmid construction by Golden Gate method. Constructs contain two connector regions flanking a GFP expression cassette. (**b**) Assembly of part plasmids into plasmids containing one gene flanked by a promoter and terminator. Each assembly is generated from three part plasmids—promoter, gene, and terminator—and a backbone plasmid. The backbone plasmid contains GFP, so that transformants containing successful assemblies can be easily distinguished from transformants containing the parent plasmid based on colony fluorescence. (**c**) Single gene-containing plasmids are assembled into repair template plasmids (containing the entire four-gene

pathway). Unique restriction enzymes flank the repair template sequence, allowing for fragment linearization preceding brewer's yeast transformation.

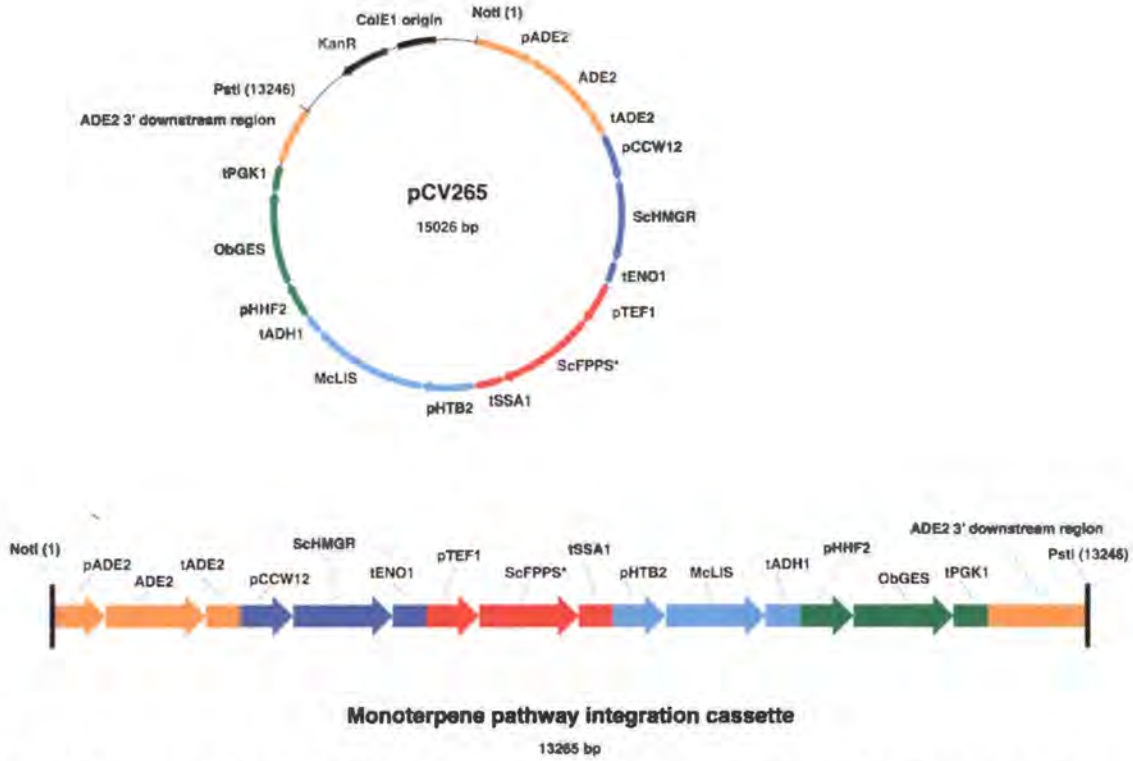

Figure 6: Schematic of the monoterpene pathway integration plasmid and linearized cassette

pCV265 contains the pathway integration cassette flanked by ADE2 homology regions. Additionally, it contains an origin of replication (ColE1 origin) and the KanR drug resistance marker for propagation and selection in *E. coli*. The NotI and PstI restriction sites are represented by solid black bars. The pCV265 plasmid is linearized before yeast transformation by digestion with corresponding restriction enzymes.

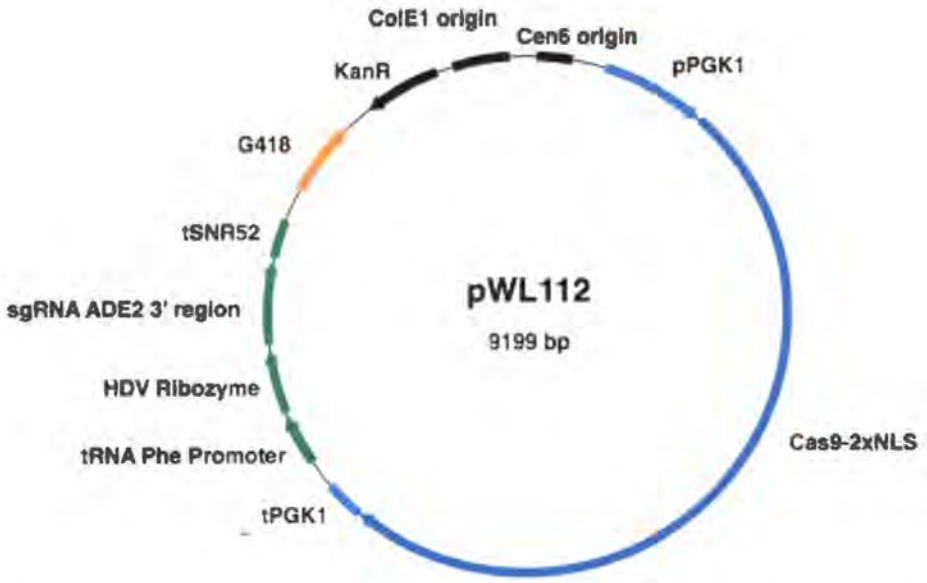

Figure 7: A schematic of the Cas9 plasmid pWL112 targeting the ADE2 3' region

pWL112 includes the following genetic elements: Cas9-2xNLS encodes a Cas9 protein with a nuclear localization tag, flanked by regulatory elements (PGK1 promoter and terminator), and the tRNA promoter, HDV ribozyme, and SNR52 terminator regulate expression of the guide RNA targeting the ADE2 3' region (inside the ADE2 terminator). Additionally, it contains an origin of replication (ColE1 origin) and the KanR drug resistance marker for propagation and selection in *E. coli*, and an origin of replication (Cen6 origin) and the G418 drug resistance marker for propagation and selection in yeast.

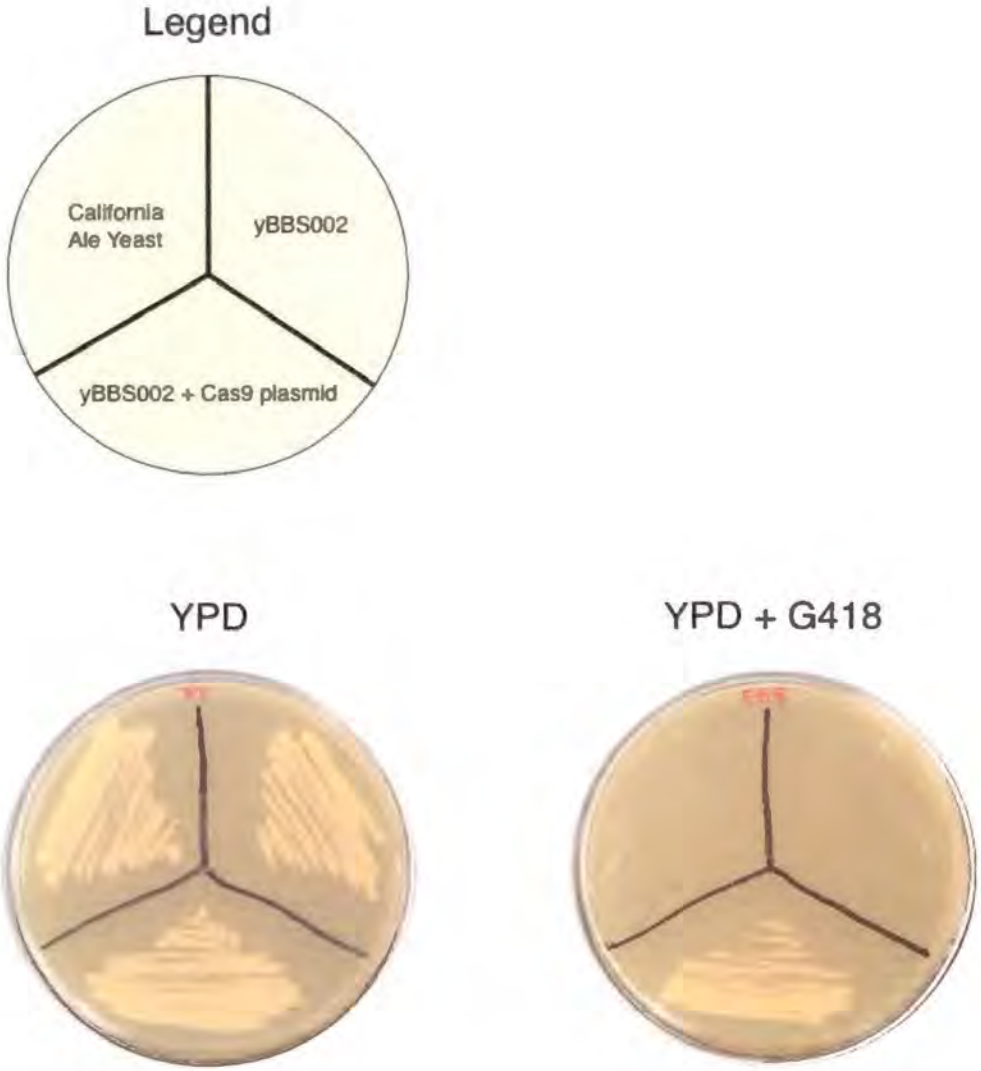

Figure 8: Confirmation of the loss of the Cas9 plasmid that conveys resistance to the antibiotic G418

The Cas9 plasmid contains the G418 drug resistance marker for selection in yeast following transformation. Following confirmation of the successful integration, the yBBS002 strain was cured of the plasmid by several passages on YPD media. Plasmid loss was confirmed by testing for growth on G418 and confirming sensitivity.

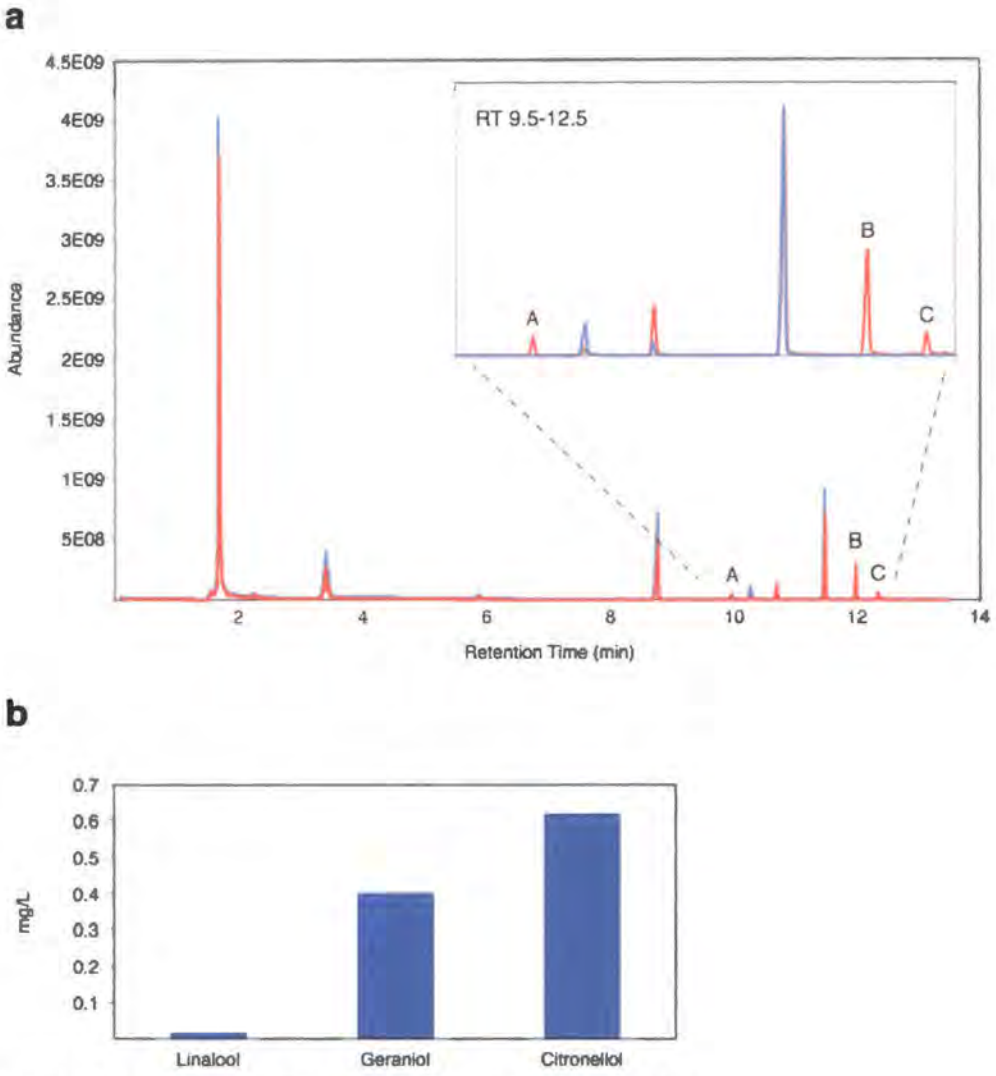

Figure 9: Gas chromatography mass spectrometry data

(a) GCMS traces from CAY (blue) and yBBS002 (red) fermentation samples. Peak A corresponds to linalool, B to citronellol, C to geraniol. Also identified in the magnified trace are phenylethyl alcohol (RT 10.3) and octyl acetate (RT 10.7), flavor molecules normally produced during fermentation and present in beer[25, 26]. **(b)** Quantification of monoterpene production by strain yBBS002.

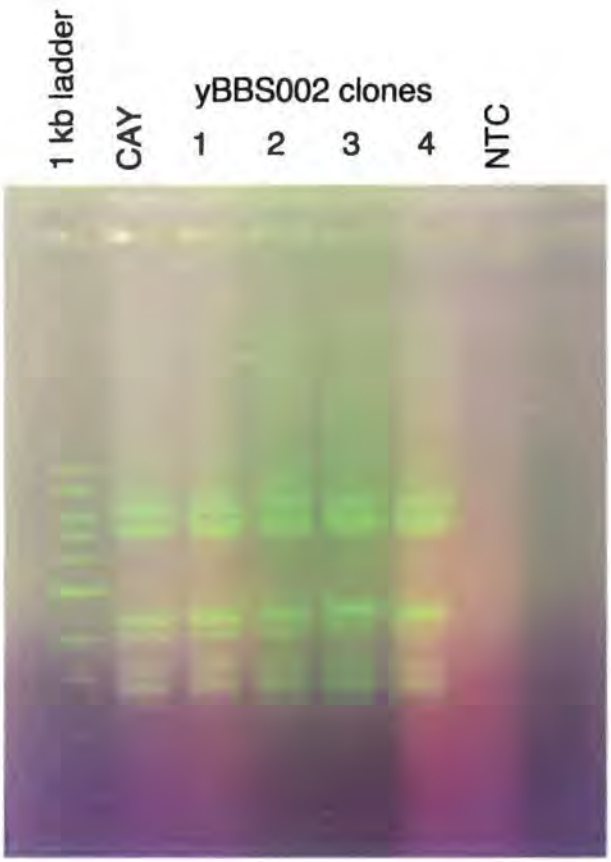

Figure 10: PCR of genetic patterns of the CAY and yBBS002 yeast strains (four clones of yBBS002 are shown)

PCR banding pattern shows that yBBS002 is genetically identical to parent strain CAY. PCR was performed using primers 5'-GTGGATTTTTATTCCAAC-3' and 5'-TCAACAATGGAATCCCAAC-3' (delta2/delta12 primer pair) on genomic DNA isolated from CAY and yBBS002 strains. Optimization of this technique (including primer sequences) is described by Legras *et al.* (2003)[27] and Schuller *et al.* (2004)[28].

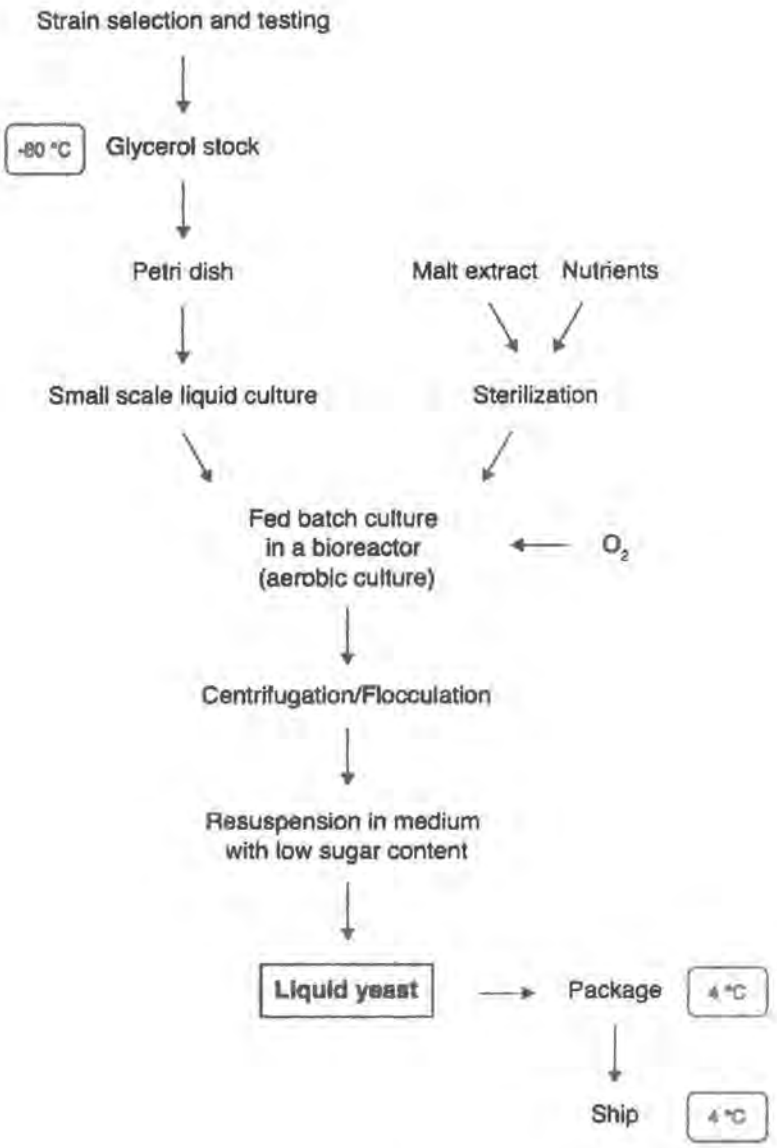

Figure 11: Flow diagram describing the manufacture of liquid yeast

Strains are stored as glycerol stocks. Frozen stocks are streaked for growth onto petri dishes containing YPD growth medium. Single colonies are used to inoculate initial small scale liquid cultures; the resulting cultures are used to inoculate large scale cultures in a bioreactor, which are grown aerobically in medium containing malt extract. Cells are separated from the medium via centrifugation or flocculation, resuspended in a medium with a low sugar content, and packaged for shipment.

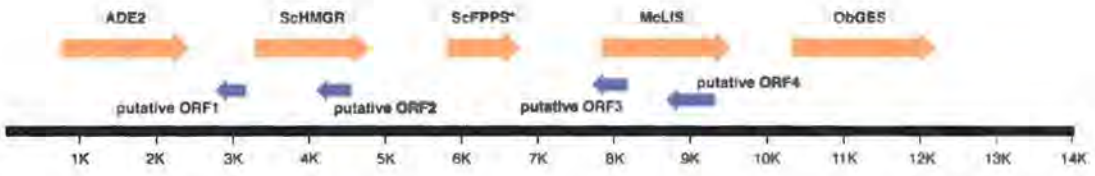

Figure 12: Putative open reading frames (ORFs) analysis

Schematic representation of putative ORFs found within the integration cassette. The chances of the putative ORFs being expressed as a stable peptide are very weak. In the unlikely event that any of the putative ORFs are translated into a stable proteins, the amino acid sequences are unlikely to be allergenic or toxicogenic based on their lack of similarity with known allergens/toxins (http://allergenonline.org). Therefore, there is no reason to believe that the yBBS002 strain will release toxic or allergenic substances as a result of the presence of unintended gene products.

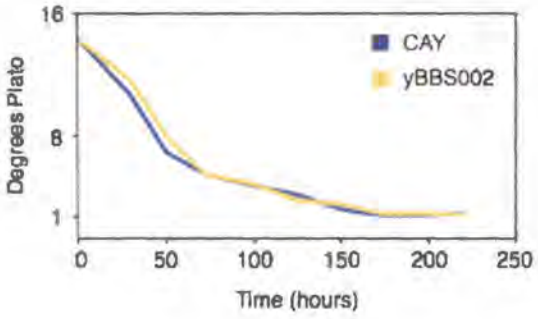

Figure 13: Fermentation rates of the CAY and yBBS002 strains

Brewing trials show that the yBBS002 strain and the CAY strain have similar fermentation rates.

Figure 14: Brewing practices and the use of a standard or yBBS002 yeast strain does not increase time required for brewing.

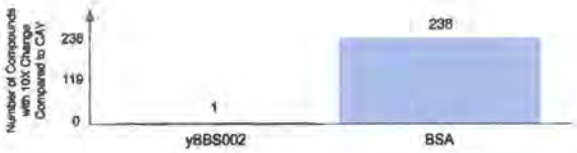

Figure 15: Metabolite differences between strains yBBS002/CAY, and between strains BSA/CSA

The number of compounds with at least 10-fold difference when CAY is compared with either yBBS002 or brewing strain BSA. While there is only 1 compound with a >10-fold change between CAY and yBBS002, there are 238 compounds with >10-fold change between CAY and BSA, demonstrating how similar the engineered strain is to the parent strain compared to differences between non-engineered brewer's yeast strains. The single compound with a >10-fold change between CAY and yBBS002 was identified as asparaginylglutamine (predicted by MSMS spectral deconvolution). This compound is a dipeptide, and is likely derived from protein degradation/metabolism.

Appendix 1: The ScHMGR gene in FASTA format

>

atggaccaattggtgaaaactgaagtcaccaagaagtcttttactgctcctgtacaaaaggcttctacaccagttttaaccaataaaaca
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Appendix 2: The ScFPPS* gene in FASTA format

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Appendix 3: The McLIS gene in FASTA format

>

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Appendix 4: The ObGES gene in FASTA format

>

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Appendix 5: The linear ADE2 deletion repair template in FASTA format

>

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Appendix 6: The linear pathway integration cassette in FASTA format

>

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Appendix 7: Putative open reading frames as a result of the integration event

ORF 1

Nucleotide sequence:

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Putative protein sequence:

MGNSKTNGFFTPEKYLYGIIQGLPPTLRGLAFIFFFLVAFYFFPAFWDLCGVLRGARGKGFPKR
KSDANSQHSKQFWTHSDFPIWFLRVLIPTRASINSMKYPHTAALVR*

ORF2

Nucleotide sequence:

atggcaccttgtggttctagaacagtaccaccaccgatggtacctacttcgatggatggcatggatacggaaattctcaaatcaccgtc
cacttctttcatcaatgttatacagttggaactttcaacattttgtgcaggatcttgtcctaatgccaagaaaacagctgtcactaaattagct
gcatgtgcgttaaatccaccaacagacccagccattgcagatccaaccaaattcttagcaatgttcaactcaaccaatgcggaaacat
cacttttaacacttttctgacaacatcaccaggaatagtagcttctgcgacgacactcttaccacgaccttcgatccagttgatggcagct
ggtttttgtcggtacagtag

Putative protein sequence:

MAPCGSRTVPPPMVPTSMDGMDTEILKSPSTSFINVIQLELSTFCAGSCPNAKKTAVTKLAACA
LNPPTDPAIADPTKFLAMFNSTNAETSLFNTFLTTSPGIVASATTLLPRPSIQLMAAGFLSVQ*

ORF3

Nucleotide sequence:

Atgcaagtcaccaaccttggtagagttgttgttttggaagatcttgtagatggagttcaagatgttcttgatttcgtccctgaagaagtaaga
caaacccaagttttgcaagtcgtcgatcaattccaattgtttggtagcttccattttagcacccaacaacttttgacttccatgatcaattcttc
ttctctttccaattgcttttcttctttgtagtggtcggtatccaaagattggatgaagtcgaaatcccaaacagatgggtgataattaccggat
cttctagtcatagatcttag

Putative protein sequence:

MQVTNLGRVVVLEDLVDGVQDVLDFVPEEVRQTQVLQVVDQFQLFGSFHFSTQQLFDFHDQF
FFSFQLLFFFVVVGIQRLDEVEIPNRWVIITGSSSHRS*

ORF4

Nucleotide sequence:

atgttgcaattgagagtggttaccatcaccatccaagtacatgaattgagcatctctacccaaattagcggcagccataactacatcac
ctctcaaatcggagttagcagccatagtagtgttcatttctttccaggcttctctaatcatgaatctaacgtgttcttctgcttcttctctggtggc
gttttgttccttcatgtagatttgaatagacttagcaacgtcacctctcttcatttcgaaaggcaaagtacccaaatcatctggcaatctaac
caacataccggacaatctgataatttcgtcgtattcgtagaaagattcgatgactggcttttctttggacttagccaacataaagtagatttg
ggaaataacagctggagcaccaatggtaatagaagcgtagttcaagtattcttccatggttggagtgtacttagtgtagtaccatttagct
tcttgcaagtaaccttcaaccaaatcaacccatgctctatgcaagaatgggatggagatgaaaccttcttctttcaagatatggtaggca
acttcactgacgaaattgtacaagacgaagtagaataa

Putative protein sequence:

MLQLRVVTITIQVHELSISTQISGSHNYITSQIGVSSHSSVHFFPGFSNHESNVFFCFFSGGVLFL
HVDLNRLSNVTSLHFERQSTQIIWQSNQHTGQSDNFVVFVERFDDWLFFGLSQHKVDLGNNS
WSTNGNRSVVQVFFHGWSVLSVVPFSFLQVTFNQINPCSMQEWDGDETFFFQDMVGNFTDEI
VQDEVE*

REFERENCES

1. Peacock, V. E., Deinzer, M. L., Likens, S. T., Nickerson, G. B. & McGill, L. A. Floral hop aroma in beer. *J. Agric. Food Chem.* **29,** 1265–1269 (1981).
2. Takoi. Screening of Geraniol-rich Flavor Hop and Interesting Behavior of beta-Citronellol During Fermentation under Various Hop-Addition Timings. *ASBC* (2014). doi:10.1094/ASBCJ-2014-0116-01
3. Mortimer, R. K. & Johnston, J. R. Genealogy of principal strains of the yeast genetic stock center. *Genetics* **113,** 35–43 (1986).
4. Crowell, A. L., Williams, D. C., Davis, E. M., Wildung, M. R. & Croteau, R. Molecular cloning and characterization of a new linalool synthase. *Arch. Biochem. Biophys.* **405,** 112–121 (2002).
5. Basson, M. E., Thorsness, M. & Rine, J. Saccharomyces cerevisiae contains two functional genes encoding 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *Proc. Natl. Acad. Sci. U. S. A.* **83,** 5563–5567 (1986).
6. Ignea, C., Pontini, M., Maffei, M. E., Makris, A. M. & Kampranis, S. C. Engineering monoterpene production in yeast using a synthetic dominant negative geranyl diphosphate synthase. *ACS Synth. Biol.* **3,** 298–306 (2014).
7. Anderson, M. S., Yarger, J. G., Burck, C. L. & Poulter, C. D. Farnesyl diphosphate synthetase. Molecular cloning, sequence, and expression of an essential gene from Saccharomyces cerevisiae. *J. Biol. Chem.* **264,** 19176–19184 (1989).
8. Iijima, Y., Gang, D. R., Fridman, E., Lewinsohn, E. & Pichersky, E. Characterization of geraniol synthase from the peltate glands of sweet basil. *Plant Physiol.* **134,** 370–379 (2004).
9. Sanchez, N. B., Lederer, C. L., Nickerson, G. B., Libbey, L. M. & McDaniel, M. R. Sensory and Analytical Evaluation of Hop Oil Oxygenated Fractions. in *Developments in Food Science* (ed. Charalambous, G.) **29,** 371–402 (Elsevier, 1992).
10. Steinhaus, M. & Schieberle, P. Comparison of the Most Odor-Active Compounds in Fresh and Dried Hop Cones (Humulus lupulus L. Variety Spalter Select) Based on GC−Olfactometry and Odor Dilution Techniques. *J. Agric. Food Chem.* **48,** 1776–1783 (2000).
11. Lermusieau, G., Bulens, M. & Collin, S. Use of GC−Olfactometry to Identify the Hop Aromatic Compounds in Beer. *J. Agric. Food Chem.* **49,** 3867–3874 (2001).
12. Irwin, A. J. VARIETAL DEPENDENCE OF HOP FLAVOUR VOLATILES IN LAGER. *J. Inst.*

Brew. **95,** 185–194 (1989).

13. Steinhaus, M., Fritsch, H. T. & Schieberle, P. Quantitation of (R)- and (S)-linalool in beer using solid phase microextraction (SPME) in combination with a stable isotope dilution assay (SIDA). *J. Agric. Food Chem.* **51,** 7100–7105 (2003).
14. America, H. G. of. HGA 2016 Statistical Packet - January 2017. Available at: https://www.usahops.org/enthusiasts/2016-hga-statistical-packet. (Accessed: 19th June 2018)
15. Food, U. S., Administration, D. & Others. CFR-code of federal regulations title 21. *Current good manufacturing practice for finished pharmaceuticals Part* **211,** (2015).
16. Kurtzman, C. & Fell, J. W. *The Yeasts - A Taxonomic Study*. (Elsevier, 1998).
17. Verma, S. K. *et al.* Chemical composition and antimicrobial activity of bergamot-mint (Mentha citrata Ehrh.) essential oils isolated from the herbage and aqueous distillate using different methods. *Ind. Crops Prod.* **91,** 152–160 (2016).
18. Gaber, R. F., Copple, D. M., Kennedy, B. K., Vidal, M. & Bard, M. The yeast gene ERG6 is required for normal membrane function but is not essential for biosynthesis of the cell-cycle-sparking sterol. *Mol. Cell. Biol.* **9,** 3447–3456 (1989).
19. Polakowski, T., Stahl, U. & Lang, C. Overexpression of a cytosolic hydroxymethylglutaryl-CoA reductase leads to squalene accumulation in yeast. *Appl. Microbiol. Biotechnol.* **49,** 66–71 (1998).
20. Reddy, L. H. & Couvreur, P. Squalene: A natural triterpene for use in disease management and therapy. *Adv. Drug Deliv. Rev.* **61,** 1412–1426 (2009).
21. Evaluation of Allergenicity of Genetically Modified Foods. Available at: http://www.fao.org/docrep/007/y0820e/y0820e00.htm. (Accessed: 14th May 2018)
22. The state of food insecurity in the world 2001. Available at: http://www.fao.org/docrep/003/y1500e/y1500e00.htm. (Accessed: 14th May 2018)
23. Organization, W. H. & Others. *Foods derived from modern biotechnology*. (Food and Agriculture Organization of the United Nations (FAO), 2009).
24. Gallone, B. *et al.* Domestication and Divergence of Saccharomyces cerevisiae Beer Yeasts. *Cell* **166,** 1397–1410.e16 (2016).
25. Äyräpää, T. OCCURRENCE OF PHENETHYL ALCOHOL IN BEER. *J. Inst. Brew.* **67,** 262–266 (1961).
26. Verstrepen, K. J. *et al.* Expression levels of the yeast alcohol acetyltransferase genes ATF1, Lg-ATF1, and ATF2 control the formation of a broad range of volatile esters. *Appl. Environ. Microbiol.* **69,** 5228–5237 (2003).

27. Legras, J.-L. & Karst, F. Optimisation of interdelta analysis for Saccharomyces cerevisiae strain characterisation. *FEMS Microbiol. Lett.* **221,** 249–255 (2003).
28. Schuller, D., Valero, E., Dequin, S. & Casal, M. Survey of molecular methods for the typing of wine yeast strains. *FEMS Microbiol. Lett.* **231,** 19–26 (2004).

Sixty-two pages of an open access publication has been removed. The removed reference is:

Denby, “Industrial brewing yeast engineered for the production of primary flavor determinants in hopped beer” *Nature Communications* volume 9, Article number: 965 (2018)
https://www.nature.com/articles/s41467-018-03293-x

From:	Rachel Li
To:	Charles Denby
Cc:	McMahon, Carrie; Viebrock, Lauren
Subject:	Re: GRN 798 - S. cerevisiae yBBS002 strain - questions
Date:	Friday, March 08, 2019 3:21:08 PM
Attachments:	image002.png GRN 798_response_to_questions_identity and manufacturing_2019-03-08.pdf SI_file.pdf

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Hi Carrie and Lauren,

Please see our answers on the attached sheet, following each question. I have also attached the supplementary information from our paper.

Please let us know if you require any further information.

Thank you very much,
Rachel

On Wed, Feb 27, 2019 at 11:55 AM Charles Denby <charles@bbsbeer.com> wrote:

> Hi Carrie,
>
> We will dig into these questions this week and get back to you next week to set up a phone call.
>
> Thanks for your help with this!!
>
> Charles
>
> On Fri, Feb 22, 2019 at 9:03 AM McMahon, Carrie <Carrie.McMahon@fda.hhs.gov> wrote:
>
> > Dear Dr. Denby,
> >
> > **REGARDING: GRN 798 (*S. cerevisiae* yBBS002 strain)**
> >
> > Since returning from the federal government shutdown, we have been working to get caught up on our evaluation of Berkeley Brewing Science's GRAS notice. We recognize that your participation in our GRAS Notice Program is voluntary and we appreciate your patience during this interruption.
> >
> > Before we can complete our evaluation of BBS's GRAS notice, we need some additional information. I have attached a few questions about the identity and manufacturing method of *S. cerevisiae* yBBS002 strain that we think are straight-forward and easily answered. Your answers to these questions will be useful in a future telephone call I would like to schedule to discuss how best to fill in a few gaps in the Dietary Exposure, Narrative, and

Supporting Data/Information sections (Parts 3, 6, and 7) of BBS's GRAS notice. The best way to fill these gaps depends on whether you already have the data in hand or whether there is relevant published literature that you can cite. I have found that a quick conversation is often the most efficient way to fill the gaps needed to bring our evaluation to closure.

I will be out of the office next week (February 25 – March 1). While I am away, if you need any clarification about our attached questions or if you would like to set up a day/time for the telephone discussion, please contact my colleague Dr. Lauren Viebrock (Lauren.Viebrock@fda.hhs.gov). She is a member of the team assigned to evaluate BBS's GRAS notice and will assist you in my absence. I've copied her on this email.

Regards,

Carrie McMahon, Ph.D.
Consumer Safety Officer

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 240-402-1202
Carrie.McMahon@fda.hhs.gov

FDA U.S. FOOD & DRUG ADMINISTRATION

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Charles Denby, Ph.D.
Berkeley Brewing Science CEO, Co-founder
(C) 206-799-2668
www.bbsbeer.com

GRN 798 – *S. cerevisiae* yBBS002 strain February 22, 2019

Questions for the notifier (Berkeley Brewing Science)

Identity

1. Is yBBS002 one of the strains described in the Denby *et. al.* (2018) Nature Communications article (or derived from one of these)? If so, which one?

    a. The published article contains information about the characterization of BBS's yeast strains that its GRAS notice does not. Because BBS does not directly state the link between yBBS002 and a specific strain in the publication, the publication data/results are of limited use in the safety evaluation of yBBS002 specifically.

    Yes, yBBS002 is strain JBEI-16652

2. Please provide a copy of the Supplementary Information (containing Figures and Tables showing experimental results from strain characterization) referenced in the Denby *et. al.* article.

    a. The published article refers to Supplementary Information concerning strain-specific characterization data that BBS's GRAS notice does not contain. Assuming the Supplementary Information contains data characterizing the yBBS002 strain, this document is relevant to BBS's GRAS conclusion.

Manufacture – Finished food-grade product

3. Are all starting/raw materials used in the manufacture of the liquid yeast product food-grade?

    a. Materials used in the cell culture and subsequent downstream processing (including antifoaming and flocculating agents, if used) should be identified and suitable for use in food processing.

    Yes, all starting/raw materials are food-grade. Malt extract is the only material used, there are no downstream processing agents.

4. Do any components of the manufacturing process (culture media, malt extract, nutrients) or finished liquid yeast product (resuspension medium) include or derive from one of the eight major food allergens? For example, we assume that malt extract is derived from wheat or barley. Is this correct?

    a. The identity of manufacturing components which may be present in the final product is important for compliance with labeling under the Food Allergen Labeling and Consumer Protection Act. FALCPA specifies eight major food allergens: milk,

eggs, fish, Crustacean shellfish, tree nuts, peanuts, wheat, and soybeans. Gluten-free labeling further specifies barley and rye.

None of the components include or derive from one of the eight major food allergens. Malt extract is derived from barley only. Yeast is grown on a Malt extract enrichment medium without milk, eggs, fish, Crustacean shellfish, tree nuts, peanuts, wheat, or soybeans.

5. Please provide specifications for the finished food-grade liquid yeast product. Specifications should include viable cell count, microbial contaminant limits, and appropriate heavy metal limits (lead, arsenic, total).

a. Food grade specifications serve two purposes. These chemically define the ingredient that is the subject of the GRAS notice and they set limits for manufacturing residues and potential contaminants that determine whether the finished product is food-grade.

Each lot is tested to be greater than 95% viable on ship date, at a concentration of 2.75 x 10 9 cells/ml and free from detectable levels of contaminants (zero colony forming units (CFUs) of bacteria or yeast detected per 250 million yeast cells). We do not anticipate dangerous levels of heavy metals.

6. Comment: BBS's reference to the use of linalool, geraniol, and citronellol in food in accordance with GMP is incorrect: BBS cites 21 CFR 211 (GMP for finished pharmaceuticals). The correct regulation for food GMPs is 21 CFR 110.

From: Rachel Li
To: McMahon, Carrie
Subject: Re: GRN 798 - additional questions
Date: Wednesday, April 17, 2019 2:20:13 PM
Attachments: image002.png

Hi Carrie,

We are working on putting answers to the questions together and should have them for you soon. We are slightly confused about the differences and outputs of the various toxin databases. Could you point me in the direction of an example notice or paper that does this analysis?

Thank you,
Rachel

On Fri, Apr 12, 2019 at 1:33 PM McMahon, Carrie <Carrie.McMahon@fda.hhs.gov> wrote:

> Dear Dr. Denby,
>
> Per our discussion, I'm relaying a few additional questions. Please don't hesitate to contact me if you need clarification. Once we have your response, we'll work to complete our evaluation of your GRAS notice for yBBS002.
>
> Regards,
>
> **Carrie McMahon, Ph.D.**
> *Consumer Safety Officer*
>
> **Center for Food Safety and Applied Nutrition**
> **Office of Food Additive Safety**
> **U.S. Food and Drug Administration**
> Tel: 240-402-1202
> Carrie.McMahon@fda.hhs.gov
>
> 
>
>
> 

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Rachel Li
Co-founder
Berkeley Brewing Science
2451 Peralta St

Oakland, CA 94607

From: Rachel Li
To: McMahon, Carrie
Subject: Re: GRN 798 - additional questions
Date: Thursday, April 18, 2019 4:32:13 PM
Attachments: image002.png

Hi Carrie,

No problem at all, (b) (6) Thank you very much for the detailed answer. I will take a look at all of this and have answers back to you early next week.

Thank you,
Rachel

On Thu, Apr 18, 2019 at 11:50 AM McMahon, Carrie <Carrie.McMahon@fda.hhs.gov> wrote:

> Rachel,
>
> First, my apologies for not including more explanation during the call or in my follow-up email last Friday. (b) (6)
> (b) (6)
>
> Dr. Viebrock provided the following examples:
>
> - UNIPROT database (Ex. GRAS Notice GRN 728, bioinformatic text in GRAS notice, and FDA response letter)
> - BLAST-P database (Ex. GRN 707, bioinformatic text is in amendment to GRAS notice (not posted on website), and FDA response letter)
> - ToxinPred database (Ex. 743, bioinformatic text in GRAS notice and FDA response letter)
> - Toxin and Toxin Target Database T3DB (Ex. GRN 736, bioinformatic text in GRAS notice, but not yet posted on FDA website, and FDA response letter)
>
> If you go to www.fda.gov/grasnoticeinventory and click on the GRAS notice number (in blue), the hyperlink will take you to a page where you can view a copy of the company's GRAS notice and FDA's response letter. For GRNs 728 and 743, you'll see that the companies provide a short summary of their analysis, results and conclusion. You'll also see in all the FDA letters that we make note of this aspect of the safety assessment.
>
> - Unfortunately, we are a little behind posting GRAS notices to our inventory, so GRN 736 isn't yet available through our website. I cannot simply provide a full copy at this time without having you submit a formal request (under the Freedom of Information Act) which will take time. I spoke with my supervisor and he agreed I could send you

the relevant page from GRAS Notice 736. See attached.

In short, we expect BBS's response to include: the database you used, the database version (or date of search), your search/alignment parameters, any significant results (and how you define significant), and your rationale for why any significant results do not change BBS's conclusion that the expressed enzymes do not raise oral toxicity concerns.

Let me know if this information doesn't answer your question.

Regards,

Carrie McMahon, Ph.D.
Consumer Safety Officer

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 240-402-1202
Carrie.McMahon@fda.hhs.gov

From: McMahon, Carrie
Sent: Wednesday, April 17, 2019 6:20 PM
To: Rachel Li <rachel@bbsbeer.com>
Subject: RE: GRN 798 - additional questions

Hi Rachel,

I'll have something for you by tomorrow. Dr. Viebrock did provide me with some example GRAS Notices. Unfortunately, I can't point you to our the examples on our GRAS

Inventory Website because either (1) we haven't posted the example notices to our website yet or (2) the bioinformatic analysis details themselves are not in the original GRAS notice but in a response to a question from FDA (and thus not on the inventory website).

Let me chat with my supervisor to see what I can do.

In the interim, I found a published study that I think will give you some practical insight to the bioinformatic approach to assessing the toxicity/allergenicity potential of proteins derived from engineered sources: https://www.sciencedirect.com/science/article/pii/S027869151730474X . Although the paper is about engineered bananas, I think there are several basic concepts that apply to your case.

For your information, the response to the bioinformatic/toxin question that we'll be looking for from BBS should be a short paragraph or so, depending on the results of your analysis.

Regards,

Carrie

From: Rachel Li <rachel@bbsbeer.com>
Sent: Wednesday, April 17, 2019 2:19 PM
To: McMahon, Carrie <Carrie.McMahon@fda.hhs.gov>
Subject: Re: GRN 798 - additional questions

Hi Carrie,

We are working on putting answers to the questions together and should have them for you soon. We are slightly confused about the differences and outputs of the various toxin databases. Could you point me in the direction of an example notice or paper that does this analysis?

Thank you,

Rachel

--
Rachel Li
Co-founder
Berkeley Brewing Science
2451 Peralta St
Oakland, CA 94607

From:	Rachel Li
To:	McMahon, Carrie
Cc:	Charles Denby
Subject:	Re: GRN 798 - additional questions
Date:	Thursday, April 25, 2019 8:37:27 PM
Attachments:	image002.png GRN 798_EDI and narrative questions_2019-04-12_answers.pdf

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Hi Carrie,

Our responses are attached. Please let us know if you require any further information. We look forward to hearing back from you.

Thank you,
Rachel

On Fri, Apr 12, 2019 at 1:33 PM McMahon, Carrie <Carrie.McMahon@fda.hhs.gov> wrote:

> Dear Dr. Denby,
>
> Per our discussion, I'm relaying a few additional questions. Please don't hesitate to contact me if you need clarification. Once we have your response, we'll work to complete our evaluation of your GRAS notice for yBBS002.
>
> Regards,
>
> **Carrie McMahon, Ph.D.**
> *Consumer Safety Officer*
>
> **Center for Food Safety and Applied Nutrition**
> **Office of Food Additive Safety**
> **U.S. Food and Drug Administration**
> Tel: 240-402-1202
> Carrie.McMahon@fda.hhs.gov
>
> U.S. FOOD & DRUG ADMINISTRATION

--
Rachel Li, PhD
Co-founder
Berkeley Brewing Science
2451 Peralta St

Oakland, CA 94607

GRAS Notice No. 000798 – yBBS002 April 12, 2019

Questions for the notifier (Berkeley Brewing Science)

DIETARY EXPOSURE

Yeast and protein: In Section 3.2.2, BBS concludes that dietary exposure to yBBS002 in finished beer will be "extremely limited." BBS's supports its conclusion by stating that the levels of yBBS002 are not detectable by optical density measurements (without providing the experiment methods, results, or the limit of detection). For FDA to evaluate BBS's conclusion, we need more information.

1. Please provide a summary of the method (including the limit of detection) and the results for the optical density measurement, noted in Section 3.2.2, which are the basis for BBS's statement that levels of yBBS002 are not detectable and therefore final concentrations are negligible in finished beer.

Beer was sampled directly from a storage vessel (keg) and placed into a cuvette, and the optical density was measured using a spectrophotometer at a wavelength of 600nm. Beer made with the parent strain CAY was used as the blank. The optical density was measured at 0.003. A culture of the parent strain of known concentration and a blank were used to calculate the limit of detection of the spectrophotometer (see Armbruster and Pry 2008, PMID 18852857). This was determined to be 0.015. Therefore, the levels of yBBS002 in finished beer are not detectable by optical density measurements, as a reading of 0.003 is below the limit of detection.

Monoterpenes flavor molecules: In Section 3.2.2, BBS concludes that dietary exposure to the monoterpene flavor molecules "should be equivalent to their consumption from beer made by traditional hopping." BBS supports its conclusion by stating that beer produced with yBBS002 will contain similar levels of linalool, geraniol, and citronellol as are present in traditionally hopped beer. However, BBS does not provide the levels of monoterpenes in beer brewed with yBBS002 or made with traditional hopping.

2. Briefly summarize the data and/or published literature that support BBS's statement (Section 3.2.2) that "beer produced with yBBS002 will contain similar levels of linalool, geraniol, and citronellol as are present in traditionally hopped beer."

Strain yBBS002 makes 0.1mg/L linalool and 0.4mg/L geraniol. Other published literature supports these levels: see Steinhaus et al 2003 (DOI: 10.1021/jf0347057), Peacock et al 1981 (10.1021/jf00108a041), and Rettberg et al 2018 (DOI: 10.1080/03610470.2017.1402574). However, heavily dry-hopped beers can contain much higher concentrations of these terpenes; our own data show that they can contain over 2

mg/L of linalool and geraniol alone.

Safety Narrative

Protein/enzyme toxicity: In Sections 6.3.3 and 6.3.4, BBS describes its bioinformatic analysis of the expressed enzymes and putative ORFs by comparing the sequences of these to allergens in the Food Allergy Research and Resource Program allergen protein database. BBS also emphasizes that the donor DNA integrated into yBBS002 strain is derived from edible organisms with a history of safe use (that is, yeast, mint, and basil) and that clarification processes will remove the bulk of the proteins, as well as large polypeptide fragments. On these grounds, BBS concludes that the allergenicity and toxicity potential of the expressed enzymes and putative ORFs is negligible.

3. Did BBS conduct bioinformatic analyses for potential toxicity of the expressed proteins? If so, which database and parameters? (The FARRP database is not a toxin database.)

a. There are several resources used for bioinformatic analyses for homology of enzymes to toxins, including: UNIPROT database homology search to see if identify homology to known toxins, BLAST-P database, ToxinPred database, Toxin and Toxin Target Database T3DB.

In order to determine whether the expressed enzymes are homologous to known toxins, we did the following: We extracted all peptide sequences in the UniProt database annotated as “toxin”, then queried these sequences with each of our expressed genes using the blastp algorithm and standard parameter settings. All hits obtained during this search had “Expect Values” (E-values) >1, indicating that the alignments were due to short random sequence identities and did not reflect homology. We therefore conclude that our expressed proteins are not homologous to any annotated toxins in the UniProt database.

4. Are the plant-derived linalool and geraniol synthases expressed in edible parts of these plants?

Yes, the synthases are expressed in the leaves of mint and basil. See Crowell et al 2002 (PMID: 12176064) and Iijima et al 2004 (PMID: 14657409).

Are there reports in the published literature of toxicity from consumption of the parts of the plants in which the mint-derived linalool synthase and basil-derived geraniol synthase present? If yes, please discuss how this does or does not impact BBS’s safety conclusion.

None that we are aware of.

Monoterpene toxicity: BBS's safety narrative for the monoterpenes rests primarily on comparison of levels in beer brewed using yBBS002 to levels in traditionally hopped beer (see Dietary Exposure question #2).

Comment: there are several published conclusions regarding the safety of linalool, geraniol, and citronellol as flavoring substances in food. BBS's safety narrative could be strengthened by inclusion of scientific expert body conclusions.

i. FDA regulations for synthetic flavoring substances and adjuvants: 21 CFR 182.60 (GRAS list including linalool and geraniol) and 21 CFR 172.515 (food additive including citronellol)

ii. JECFA ADI group ADI (1997) for citral, geranyl acetate, citronellol and linalool is 0 to 0.5 mg/kg bw