OMEGA YEAST

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GRAS Notification Program
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

Dear Sir or Madam:

We are hereby submitting one hard copy of a new Generally Recognized as Safe (GRAS) Notification in accordance with Title 21 C.F.R. §170.30, for Omega Yeast Labs *Saccharomyces cerevisiae* strain OYR-481. Omega Yeast Labs, LLC proposes the use of OYR-481 in brewing to improve beer quality and efficiencies in brewing through the reduction of the common off flavor, diacetyl. OYR-481 with enhanced AldB activity will directly convert alpha-acetolactate produced by the yeast into acetoin, bypassing the spontaneous formation of diacetyl when alpha-acetolactate accumulates in the beer. Omega Yeast Labs has determined through scientific procedures that the OYL-481 strain is generally recognized as safe for use in the brewing industry to improve beer quality and efficiencies in brewing through the reduction diacetyl.

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

Thank you for your consideration.

Sincerely,

[redacted]

Lance Shaner

Owner, Omega Yeast

GRAS Determination for *Saccharomyces cerevisiae* strain OYR-481

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

DATE: February 15th, 2024

List of Acronyms

CRISPR – clustered regularly interspaced short palindromic repeats
sgRNA – small guide RNA
PAM – protospacer adjacent motif
bp – base pair
YPD – yeast peptone dextrose

§ 170.225 Part 1, GRAS Notice: Signed Statements and Certification

(1) GRAS Notice Submission

Omega Yeast Labs, LLC., in accordance with 21 CFR § 170.225(c)(1), has determined that its *Saccharomyces cerevisiae* expressing a gene encoding an acetolactate decarboxylase (AldB) from *Brevibacillus brevis* is Generally Recognized as Safe ("GRAS") for the intended food application in accordance with Subpart E of 21 CFR § 170.

(2) Name and Address

Omega Yeast Labs, LLC
4720 W Pensacola Ave
Chicago, IL 60641
USA

(3) Name of Notified Substance

The name of the notified substance is a brewing yeast strain of *S. cerevisiae* that has been genetically edited using the CRISPR-Cas9 system to express a gene encoding an acetolactate decarboxylase (AldB) from *Brevibacillus brevis*. This novel yeast strain is called OYR-481.

(4) Intended Use in Food

The OYR-481 strain was developed by Omega Yeast Labs to express the *B. brevis* aldB gene encoding an acetolactate decarboxylase (AldB) in fermentation to mitigate the production of common off flavor diacetyl in beer fermentations. *S. cerevisiae* produces many aroma-active metabolites that contribute to the complexity of a beer's flavor including phenolics, esters, carbonyl compounds, higher alcohols, aldehydes and sulfur compounds. Some of these metabolites are considered undesirable and flavor defects in beer. Once such compound, diacetyl, is produced throughout fermentation through the spontaneous decarboxylation of alpha-acetolactate in beer. Brewing yeast uptake diacetyl from the beer and reduce it into acetoin and then 2,3-butanediol, which are flavor inactive at the concentrations produced in beer fermentations. Diacetyl is problematic in some brewing strains with elevated production and also can accumulate as a result of re-fermentation initiated by dry hopping. When diacetyl is above flavor thresholds, it leads to production delays and can even result in product recalls if it forms in after the beer is released to the market. Thus, diacetyl is a commonly observed flavor defect that brewers take extra precaucciouns to keep below flavor threshold finished beer.

The AldB enzyme from *B. brevis* is GRAS and is commercially available as an exogenous enzyme to add directly to beer. This AldB enzyme reduces alpha-acetolactate directly to acetoin, preventing the production of diacetyl. There are many benefits to expressing the AldB enzyme in brewing yeast to reduce diacetyl rather using the exogenous enzyme. It is cost effective, avoids added steps to directly add the enzyme into the fermentation vesse, elevates the need for an entirely separate supply chain to produce the exogenous AldB enzyme and when the AldB enzyme is expressed in the yeast it is directly coupled to the production of alpha-acetolactate within the cell. Lastly, as the yeast cells are removed at the end of fermentation, the AldB enzyme stays within the cell and does not remain in the finished beer.

The OYR-481 yeast strain is intended for use as a commercial liquid yeast culture for the production of fermented beverages in accordance with Good Manufacturing Practices (GMPs). This liquid yeast strain performs alcoholic fermentation in the same manner as its host strain and other traditional brewing strains. OYR-481 provides a well-suited brewing yeast strain for american craft ales and lagers, especially dry-hopped beers and other "clean" beer styles.

(5) Statutory Basis for GRAS Determination

Omega Yeast Labs, LLC has determined that the OYR-481 yeast strain is "Generally Recognized As Safe" (GRAS) for the use as a starter culture in alcoholic beverage fermentation. This determination is based on scientific procedures and conforms to the regulations in accordance with 21 CFR § 170.30(a) and (b). Based on the design of OYR-481, it is substantially equivalent to the host strain, with the sole difference being the expression of the *B. brevis aldB* gene encoding the AldB enzyme. The resulting beers have reduced levels of the off flavor diacetyl similar to beers produced with high quality standards. The precise insertion of the *aldB* gene at the *ADE2* locus means there is a negligible chance of unintended effects in the OYR-481 strain.

(6) Premarket Approval Statement

Omega Yeast Labs further asserts that the use of the OYR-481 yeast strain ingredient, as described herein, is exempt from the pre-market approval requirements of the Federal Food, Drug, and Cosmetic Act, based on a conclusion that the substance is GRAS under the conditions of its intended use.

(7) Availability of Information

The data and information that serve as the basis for this GRAS determination, as well any information that has become available since the GRAS determination, will be sent on request, or are available for the FDA's review and copying during customary business hours at the following address:

Omega Yeast Labs, LLC
Attention: Lance Shaner
4720 W Pensacola Ave
Chicago, IL 60641
USA

(8) Data and Information Confidentiality Statement

None of the data and information in the GRAS notice are exempt from disclosure under the Freedom of Information Act, 5 U.S.C. 552.

(9) GRAS Certification

To the best of our knowledge, this GRAS notice for *Saccharomyces cerevisiae* OYR-481 and its use in fermentation of alcoholic beverages in accordance with Good Manufacturing practices (cGMP) is a complete, representative and balanced submission that includes both favorable and unfavorable information known to Omega Yeast Labs, LLC and pertinent to the evaluation of the safety and GRAS status of the use of the strain. Recent reviews of the scientific literature revealed no potential adverse health concerns.

(10) Name/Position of Notifier

[redacted] 2/15/2024

Lance Shaner, Ph.D. Date
Co-owner and Founder
Omega Yeast Labs, LLC

§ 170.230 Part 2, Identity, Method of Manufacture, Specifications, 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 OYR-481. This strain was modified with CRISPR-Cas9 to introduce the *aldB* gene from *B. brevis* for the expression of the acetolactate decarboxylase (AldB) enzyme in beer fermentations. The only non-native DNA introduced with this modification is the DNA encoding the amino acid sequence of the AldB enzyme. Technical details regarding the modification are detailed in sections below. The use of OYR-481 in brewing prevents the accumulation of the diacetyl off flavor, ensuring levels are consistent with those found in high quality beer.

2.2 HOST MICROORGANISM

The host yeast strain, OYL-004, is an industrial brewing strain of *S. cerevisiae* that is widely used in commercial beer production of American craft beers, including modern American hoppy beer styles. OYL-004 is commonly referred to by American craft brewers as "Chico" and sold by multiple commercial yeast suppliers under the names BRY-96, WLP001, WY1056 and US-05.

2.2.1. History of use

S. cerevisiae is widely used in industrial fermentation of many food products. It is in the air that we breathe and it grows naturally on many foods that we eat regularly. It is likely that most humans are exposed daily to live *S. cerevisiae* through consumption of wine, beer, fruits, salads, and cheese and other dairy foods, as well as other traditional and ethnic fermented foods that contain the yeast. This yeast species has been used in food production for over five thousand years, with common industrial applications including baked goods, beer, wine, cider, sake, spirits, coffee and chocolate (Boekhout and Robert, 2014).

S. cerevisiae serves as a model organism for scientific research. Over 30 years of yeast research has contributed to our fundamental understanding of cell and molecular biology. The *S. cerevisiae* genome size is small, is highly homologous with the human genome, genetics are very tractable, and DNA repair pathways are robust allowing for ease of accurate genetic manipulation (Botstein and Fink, 2011). A long history of research and industrial use has indicated that *S. cerevisiae* is safe for use in food and beverage manufacturing (Boekhout and Robert, 2014).

There are over 150 *S. cerevisiae* strains that are commercially sold as brewing yeast. These strains have been isolated from brewery and farmhouse origins, where the re-pitching or backslopping practices allowed for the propagation and selection of unique brewing characteristics (Gallone et al., 2016). The origin of the host strain, OYL-004, traces back to the Ballantine Brewery in Newark, NJ. It is a widely popular ale strain used by many US craft breweries for American craft beers, including modern American hoppy beer styles.

The FDA considers *S. cerevisiae* and several derived products safe for consumption. Indeed, FDA has approved dried yeast as an ingredient for food (21 C.F.R. §172.896), and Baker's yeast extract has been affirmed by the FDA as a GRAS flavoring agent and adjuvant (21 C.F.R. §184.1983). The FDA has also approved various yeast-derived products for their use in food. These include Baker's yeast protein (21 C.F.R. §172.325), Yeast-malt sprout extract (21 C.F.R. §172.590) and Baker's yeast glycan (21C.F.R. §172.898). *S. cerevisiae* is also considered GRAS as noted in several GRAS Notices (GRNs) that received "no questions" letters from the FDA (e.g., GRNs 120, 175, 239, 260, 284, 350, 353, 798, 841, 928) (for an up-to-date list of modified and unmodified *S. cerevisiae* notifications, see the Current list of S. cerevisiae GRAS Notices).

2.2.2. Taxonomy

The OYL-004 host strain's taxonomy has been confirmed to be *S. cerevisiae* by multiple whole genome sequencing studies. Furthermore, whole genome sequencing of OYL-004 confirms that it is closely related to other Belgian/ German/ English/ American "Beer 2" industrial brewing strains (Gallone et al., 2016).

2.2.3. Characteristics

The host strain exhibits standard industrial brewing characteristics. It is maltose-fermenting, and is easily propagated in wort produced from malted barley. Fermentations with this strain produce a desirable ale fermentation profile attributed to a neutral ester profile. This strain sporulates, but exhibits low spore viability and thus is not well-suited for traditional breeding strategies.

2.3. DONOR ORGANISMS

2.3.1. Taxonomy

Saccharomyces cerevisiae

The history of the use of *S. cerevisiae* is reviewed in Section 2.2.1 above. The promoter and terminator regulatory elements that are included in OYR-481 are derived from the highly characterized S288C strain of *S. cerevisiae* that serves as the reference sequence strain in the Saccharomyces Genome Database (https://yeastgenome.org/).

Brevibacillus brevis

The donor organism for the DNA encoding the acetolactate decarboxylase, is *B. brevis*. The *aldB* gene is the only DNA sourced from *B. brevis* and a full safety evaluation has been previously supported for the use of the *B. brevis* AldB enzyme in beer fermentaitons (GRN 587).

2.3.2. Genetic material synthesized based on sequences from donor organisms

The *aldB* gene

The *aldB* gene is derived from *B. brevis* and encodes an acetolactate dehydrogenase, also referred to as AldB throughout this dossier. The AldB enzyme of *B. brevis* has been characterized as necessary and sufficient for decarboxylation of alpha-acetolactate to acetoin, bypassing the production of diacetyl in alcoholic beverage fermentation (Diderichsen et al. 1990).

The *aldB* gene encoding the *B. brevis* AldB enzyme was codon optimized for expression in *S. cerevisiae*, and was synthesized as a linear DNA fragment including an *S. cerevisiae* promoter and terminator.

Native *S. cerevisiae* Promoter and Terminator Sequences

The *aldB* gene is heterologously expressed and under the control of native *S. cerevisiae* promoter and terminator sequences. These sequences were obtained from the S288C reference genome.

2.4. THE MODIFIED MICROORGANISM

2.4.1. Final construct used in the CRISPR-Cas9 strategy

2.4.1.1. Construction strategy

To generate OYR-481, the *aldB* expression cassette was targeted to the *ADE2* gene using CRISPR-Cas9 gene-editing technology, using a two step process of *ADE2* disruption and then *aldB-ADE2* repair (Mans et al., 2015).

Briefly, Cas9 (bacterial RNA-guided endonuclease) was expressed and targeted to the *ADE2* loci with a small guide RNA (sgRNA) that is homologous to the sequence surrounding the position 38-58 bp in *ADE2*. In addition, a protospacer adjacent motif (PAM) sequence (NGG) is in close proximity, which is crucial for the correct guidance of Cas9 to its target. Once recruited, Cas9 generates a double strand break. A repair template with homology to DNA surrounding the break is supplied in excess and is utilized by the yeast cell's endogenous homologous recombination pathway for repair. Once the repair occurs, the sgRNA targeting sequence is disrupted with the repair sequence and thus Cas9 is unable to efficiently target the *ADE2 locus*. Successful CRISPR-Cas9 targeting results in the editing of each *ADE2* loci of the tetraploid recipient strain, OYL-004.

In the first step of the construction strategy, disruption of the *ADE2* gene results in the accumulation of a red pigment in the yeast vacuole. This allows for transformants to easily be screened through red/white colony appearance. The *ADE2* disruption sgRNA targets +38 bp into the ADE2 coding region, and the donor repair oligonucleotide (90 bp) includes 45 pb homology to the ADE2 promoter (-600 bp) and coding region (+58 bp). This results in a 652 bp deletion in the promoter and start of the *ADE2* gene and a nonfunctional *ade2*. At this step, no DNA has been introduced into the genome, only disruption scar at the *ADE2* promoter/start region resulting repair (Figure 1B). The second step of the construction strategy, repair of the *ade2* locus, an *ade2* repair sgRNA targeted the disruption scar. This sgRNA was screened for off target sites, and confirmed that it does not target any other site in the *S. cerevisiae* genome. The repair template for this disruption included the native *ADE2* promoter/start region to repair the *ade2* locus, as well as the *aldB* gene expression cassette (1965 bp total). The resulting modification included the insertion of the *aldB* gene cassette 602 bp upstream of the ADE2 start codon (Figure 1C). The second step, was confirmed by a return to white colony color indicating repair of the *ADE2* gene, as well as PCR and sequencing confirmation for the specific insertion of the *aldB* gene cassette.

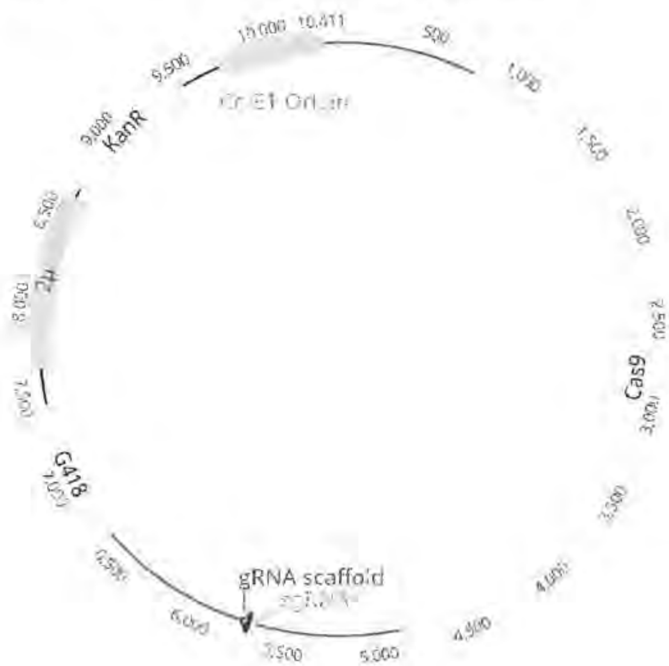
Figure 1: Schematic of the modifications resulting from the CRISPR-cas9. A. Native *ADE2* locus. B. Resulting *ade2* disruption from step one of the CRISPR-cas9 strategy. B. Resulting *aldB-ADE2* repair resulting from step two fo the CRISPR-cas9 stragety.

No genes encoding virulence factors, toxins or enzymes involved in the synthesis of mycotoxins, or any other toxic substances are expected based on our knowledge of these strains, the *aldB* sequence, the native promoter and terminators, and the combination used in the *aldB* expression cassette. The nucleotide sequences of the insertion sites and flanking regions were examined for potential open reading frames. Nineteen open reading fromes were identified (NCBI ORF finder). A bioinformatics search of the 19 potential open reading frames spanning the site of insertion (*ADE2*) and the inserted DNA (*aldB expression cassette*) was performed with NCBI BLASTp (http://www.ncbi.nlm.nih.gov/BLAST/) on January 22nd, 2024. The criteria used for BLASTp were default parameters: BLOSUM92 scoring matrix, Word size 5, Expect value 0.05, hitlist 100, Gapcosts 11,1, window size 40, threshold 0. BLAST results for each of the 19 open reading frames were manually evaluated for any suggested toxicity and a search for the word "toxin" was performed on each list of subject proteins identified among the open reading frames. None of the similar proteins identified of these BLAST searches were known toxins, leading us to conclude that the *aldB* cassette insertion does not raise any oral toxicity concerns.

2.4.1.2. Generating the CRISPR-Cas9 plasmids and repair templates

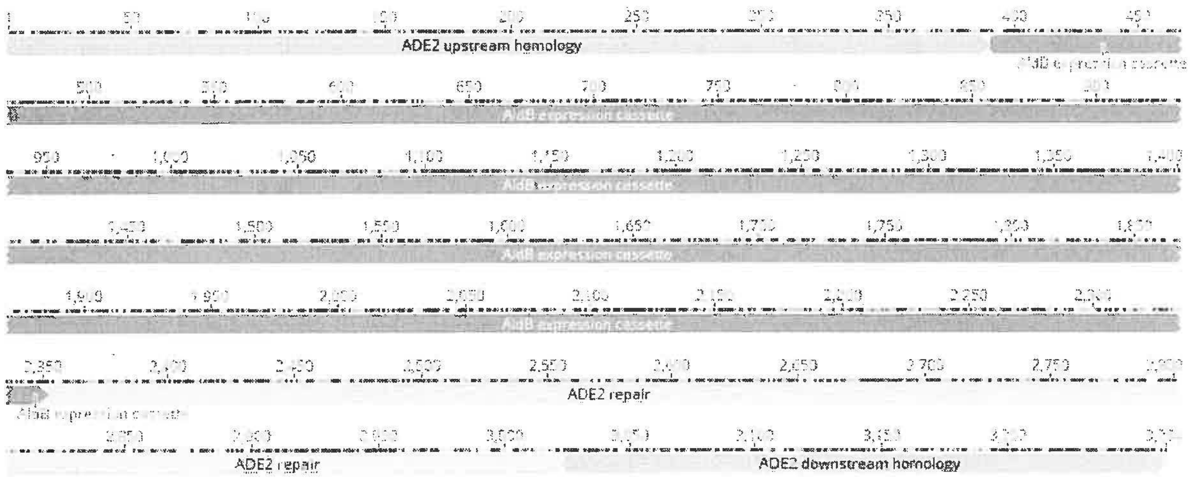
The final DNA constructs used for transformation of the host organism are the *ADE2* disruption CRISPR plasmid, *ade2* repair CRISPR plasmid, the oligonucleotide *ADE2* disruption repair template and *aldB-ADE2* repair template. To generate the CRISPR plasmids, the targeting sgRNA was cloned into a yeast shuttling vector containing Cas9 regulated by *S. cerevisiae PGK1* promoter and terminator. The yeast shuttling vector was digested with BsmBI. Forward and reverse oligonucleotides were synthesiszed with the sgRNA sequence and homology to the BsmBI digested ends. These oligonucleotides were annealed, phosphorylated and ligated into the BsmBI yeast suttling vector resulting in an sgRNA expression cassette containing the tRNA phe promoter, tRNA phe, sgRNA (*ADE2* disruption or *ade2* repair), and the tSRN52 sequences. The parent yeast shuttling vector also contains the *KanMX* gene, and thus successful gap repair results in G418-resistant colonies in *S. cerevisiae*. The two CRIPSR plasmids were confirmed through full plasmid sequencing. The *ADE2* disruption and *ade2* repair CRISPR plasmids allow for co-expression of Cas9 and sgRNA. The plasmid schematic is provided in Figure 2.

Figure 2: Schematic of CRISPR-Cas9 plasmid



The ADE2 disruption oligonucleotide was synthesized by Integrated DNA technologies, Inc (Coralville, Iowa) as a duplex oligonucleotide. The oligonucleotide contained 45 bp homology to the ADE2 promoter region 600 bp upstream of the start site and 50 bp downstream of the start site. The *aldB* expression cassette was constructed by Integrated DNA Technologies, Inc (Coralville, Iowa) as a gBlock. The gBlock included 390 bp homology to the *ADE2* promoter, 1965 pb encoding the *aldB* gene cassette, 600 bp of the disrupted ADE2 promoter/start and 240 bp homology to the ADE2 gene. The resulting aldB-ADE2 repair template was verified on both strands through Sanger sequencing. The resulting schematic is supplied in Figure 3.

Figure 3: Schematic of *aldB-ADE2* repair template



2.4.1.3. Detailed description of the final CRISPR plasmids and repair template

The *ADE2* disruption and *ade2* repair CRISPR plasmids are 10,411 bp long. The sole difference being the sgRNA sequence cloned into the yeast shuttling vector. The sequence contains the Cas9 ORF flanked by the ScPGK1 promoter and terminator (763-4,866 bp), the *ADE2 or ade2* sgRNA expression cassette containing the tRNA phe promoter, tRNA phe, *ADE2 or ade2* sgRNA, the G418 resistance gene for G418-selection in *S. cerevisiae* (6,628-7,437 bp), the tSRN52 (5,678-5,773 bp) multi-copy selfish DNA element (7,644-8,626 bp), the *KanR* gene for Kanamycin-selection in *E. coli* (8,684-9,499 bp), and the ColE E. coli origin of replication (9,724-10,312 bp). The plasmid map is provided in Figure 2 above. The *ADE2* disruption oligonucleotide contains the sequence from -657 to -599 bp in the *ADE2* promoter, and 54 to 92 bp of *ADE2*. The *ADE2-aldB* repair template includes *ADE2* upstream homology (1-390 bp), The *aldB* expression cassette (391-2,352 bp), *ADE2* repair (2,353-3,024 bp) and the *ADE2* downstream homology (3,025-3,264 bp). The schematic of the repair template containing *aldB* expression cassette is provided in Figure 3 above.

2.4.2. The transformation event

2.4.2.1. Genetic material used for the transformation method

The *ADE2* disruption CRISPR plasmid and *ADE2* disruption oligonucleotide repair template were the only DNA sequences used for the first step in generating *ade2*, resulting in easy confirmation by red colony morphology. The *ade2* repair CRISPR plasmid and *aldB-ADE2* repair template were the only DNA sequences used in the second step in generating the final OYR-481 strain with an inserted *aldB*-expression cassette at the *ADE2* locus.

2.4.2.2. Screening method for transformants

Logarithmically growing cultures of OYL-004 were transformed with 500 ng of the *ADE2* disruption CRISPR plasmid and 25 µg of the *ADE2* disruption oligonucleotide using standard lithium acetate transformation protocol.[10] The transformation reaction was outgrown in

YPD overnight and plated onto YPD-G418 agar plates to select for successful transformants. Colonies were restruck to YPD-G418 agar plates to confirm resistance and then individual transformants were screened by a red color change and validated by colony PCR. Briefly, yeast genomic DNA was prepared by lithium acetate-SDS method and primers specific to the upstream and downstream region of *ADE2* disruption were used to identify transformants with the successful CRISPR-Cas9 editing event. Confirmed *ADE2* disrupted isolates were outgrown to allow spontaneous plasmid loss of the *ADE2* disruption CRISPR plasmid and colonies that were red and sensitive to G418 were used for the second transformation event. The same procedure above was repeated using 500 ng of the *ade2* repair CRISPR plasmid and 25 ug of the *aldB-ADE2* repair template. From this transformation, white colonies were isolated confirming the *ADE2* repair and followed by colony PCR. Briefly, a PCR product of 1166 bp resulting from primers within the *aldB* expression cassette and downstream of the repair template within the *ADE2* coding region (356-379 bp) were used to verify the correct insertion.

2.4.3. Genetic characterization of the modified microorganism

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

Successful transformants were inoculated into liquid YPD, grown to saturation and subsequently diluted and plated onto YPD agar plates. Once individual colonies were observed, YPD plates were replica-plated to YPD-G418. Colonies that lost the *ADE2/ade2* CRISPR plasmid showed G418 sensitivity and were re-struck to YPD and YPD-G418 to confirm the loss of the antibiotic resistance gene (Figure 4).

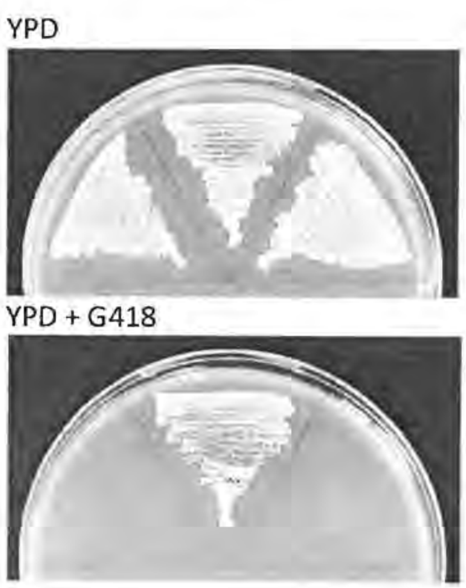


Figure 4: Loss of *ADE2* targeting CRISPR-Cas9 plasmids confirmed by loss of G418-antibiotic resistance. Strains from left to right: OYL-004, transformant before plasmid loss, and OYR-481.

2.4.3.2. PCR to confirm the *aldB-ADE2* insertion

Colony PCR of the resulting isolates was performed to amplify the insertion at *ADE2*. The PCR product was predicted to be 1166 bp. Confirmation of the 1166 bp insertion is reported in Figure 5.

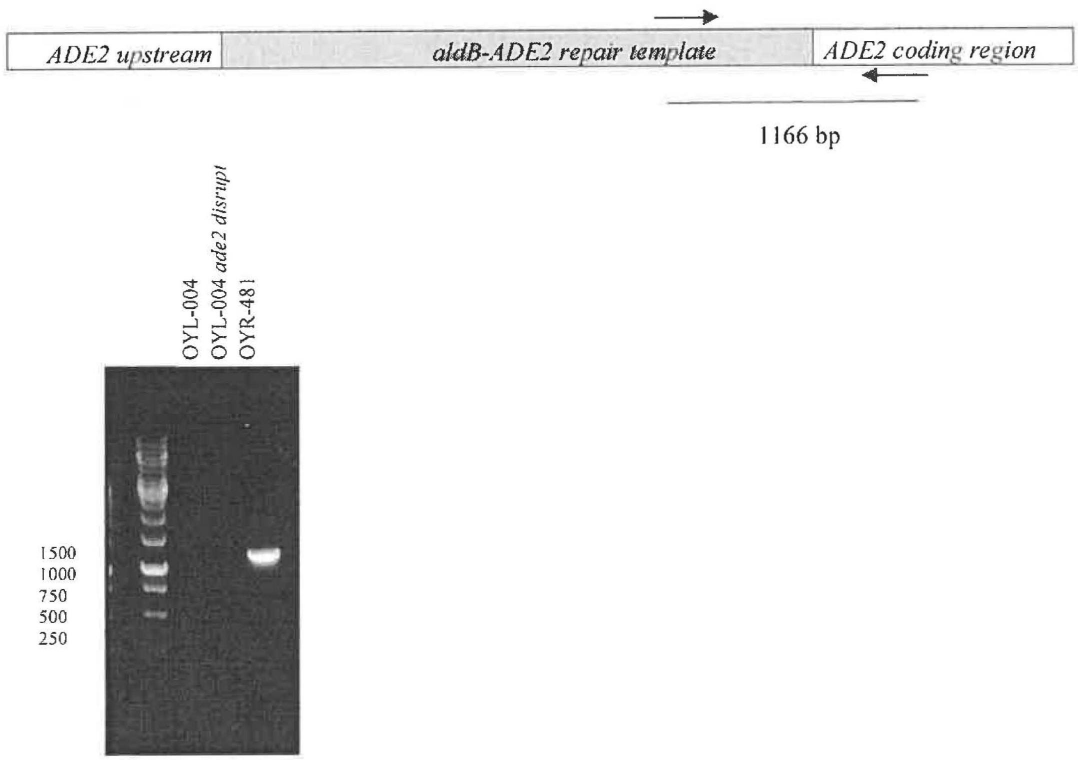


Figure 5: Colony PCR results confirming the targeted CRISPR-Cas9 editing to insert *aldB*-expression cassette at *ADE2*

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

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 δ12 (5'-TCAACAATGGAATCCCAAC-3') and δ21 (5'-CATCTTAACACCGTATATGA-3') to amplify these δ sequences, we observed identical PCR fingerprints and have verified the genetic relationship between the host strain OYL-004 and OYR-481 (Figure 6). This strategy is widely used to differentiate between industrial strains of *S. cerevisiae* (Legras and Karst, 2003). The interdelta PCR experiments were performed after serial passages on YPD agar plates, confirming that the genome is stable across generations. Furthermore, the expected genotype and phenotype of OYR-481 remains unchanged when propagated through the liquid yeast manufacturing process (Figure 7) representing greater than 30 cell divisions. The OYR-481 strain is not expected to be any more likely to undergo genomic rearrangement events within the normal environmental conditions of fermenting beer then the parent OYL-004 strain.

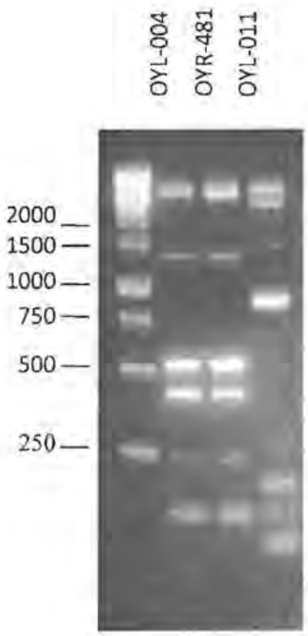


Figure 6: Interdelta PCR patterns of the OYL-004 and OYR-481 yeast strains

2.5. METHOD OF MANUFACTURE OF THE MODIFIED MICROORGANISM

2.5.1. Manufacturing of OYR-481

Propagation of the OYR-481 does not require any additional selection or alterations to the propagation methods of the host strain OYL-004 or any other industrial brewing strains. The manufacturing of OYR-481 is identical to that of all liquid yeast used in brewing (Stewart, 2017). All raw materials are food grade and free of the nine major food allergens as defined by the U.S. Food Allergen Labeling and Consumer Production Act of 2004 and the Food Allergy Safety, Treatment, Education, and Research Act of 2021. Packaged product is >95% viable on ship date, at a concentration of 2-3 x 10^9 cells/ml and free from detectable levels of contaminants (zero colony forming units of bacteria or yeast detected per 40 million yeast cells). A summary of the methods used in manufacturing of OYR-481 can be found in Figure 7.

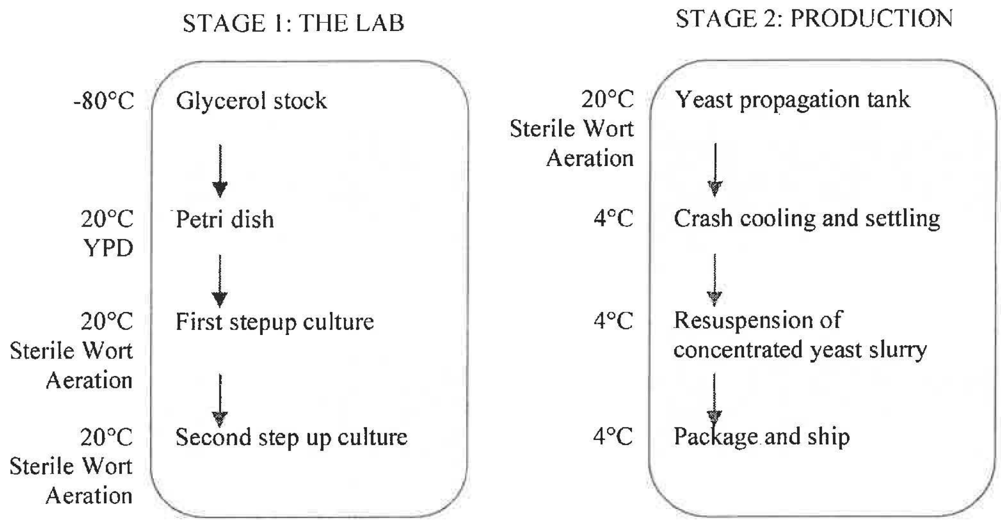


Figure 7. Flow diagram of liquid yeast manufacturing. Strains are started from glycerol stocks by streaking directly to YPD growth media plates. The YPD colonies are inoculated into the first step up culture. After reaching saturation, this culture is inoculated into the second step up culture. The second step up culture is grown to saturation and brought to the production facility to inoculate the propagation tank. This large-scale propagation is the final stage. When the culture has depleted all fermentable sugar, the temperature is lowered to 4°C and the cells are separated through natural processes of flocculation and settling. The propagation media is decanted and the remaining yeast cell mass is resuspended as a concentrated liquid slurry. This concentrated yeast slurry is packaged and shipped to consumers.

2.5.2. Raw Materials

All raw materials are standard food grade ingredients used in the manufacturing of liquid brewing yeast. These ingredients are free of the nine major food allergens as defined by the U.S. Food Allergen Labeling and Consumer Production Act of 2004 and the Food Allergy Safety, Treatment, Education, and Research Act of 2021. All processing aids used in the manufacture of S. cerevisiae OYR-481 are food grade, concluded to be GRAS for their respective uses, and used in a manner consistent with current GMPs.

Heavy metals are potential risks identified in agriculture ingredients, as well as being present in certain water systems. Omega Yeast Labs controls these risks through cGMPs and other pre-requisite programs, requiring suppliers for agricultural ingredients to be verified by Omega Yeast and each lot of agricultural ingredient is accompanied by a Certificate of Analysis to be within food grade standards for heavy metals, herein defined as < 1.0 ppm lead, < 0.5 ppm arsenic, < 0.03 ppm cadmium, and < 10 ppm (as Pb) total heavy metals. Process water used in the facility is supplied by the municipality of Chicago, and monitored by our facility through collecting annual water quality reports from the city as well as submitting a process water sample to an external laboratory for yearly analysis for total metals (lead with a limit of < 1.1 ppb and mercury with a limit of <0.22

ppb). Thereby controlling the raw material and water inputs through cGMPs, heavy metals are not introduced during the manufacturing of OYR-481.

2.5.3. Lab Stage

OYR-481 is inoculated onto nutrient agar plates from frozen stock cultures maintained at -80°C in glycerol. The yeast colonies are transferred with a sterile loop from the nutrient agar plate into sterilized flasks of propagation medium and grown with aeration at 20°C to saturation. These starter cultures are inoculated into larger sterilized flasks of propagation medium and grown to cell numbers that are sufficient for the inoculation of a yeast propagation tank.

2.5.4. Fermentation and Recovery

The yeast from the lab is inoculated into propagation tanks in production. The propagation is aerated and grown at 20°C. Once fermentation is completed, the temperature is lowered to 4°C for settling. The yeast slurry is resuspended to a slurry concentrate of 30% yeast solids (percent pellet weight to total volume). The slurry concentrate is transferred to sterile packaging and stored at 4°C until used to inoculate a beer fermentation.

To prevent contamination of foreign microorganisms, the propagation medium is sterilized and all equipment is carefully cleaned and sanitized. The propagation tanks are cleaned in place with acid and base and rinsed to neutral pH. The cleaning is validated with microbiological testing and ATP swabs. The tanks are then sanitized with food grade sanitizer prior to filling with sterile propagation medium. The propagations are sampled throughout for microbiological controls and the yeast percent solids is determined at the end of propagation to insure proper and consistent growth.

2.6. APPLICATION AND USE LEVELS

We recommend that brewers use approximately 1 million cells of OYR-481 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.

The FDA has previously had no questions regarding this recommendation (GRN No 798). Dry yeast substances used in beer and wine fermentations (GRN No 841, GRN No 120) also have recommended use levels that are consistent with the proposed use level. There is a self-limiting use for our product as noted in "Part 4", as brewers would incur significant fermentation performance and quality issues creating a self-limiting use for GMP compliance.

2.7. PRODUCT SPECIFICATIONS

The specifications for liquid yeast are measured on every batch and subsequently approved for release by Quality Control. The liquid yeast slurry is plated on microbiological growth mediums to detect contaminating bacteria and wild yeast. Any observation of growth results in the product being discarded. At the end of fermentation, cell growth is monitored by percent yeast solids and anything under 3% is discarded. The growth medium and liquid yeast product does not contain any of the nine major food allergens as defined by the U.S. Food Allergen Labeling and Consumer Production Act of 2004 and the Food Allergy Safety, Treatment, Education, and Research Act of 2021. Product specifications are listed in the Table 1. The product specifications are ensured using analytical methods provided by the American Society of Brewing Chemists (ASBC) Methods of Analysis. Percent Solids by (Yeast 5 ASBC), Viable Count and Yeast Viability by (Yeast 3 ASBC) and (Yeast 4 ASBC) and Total Bacteria and Wild Yeast by (Microbiological Control 2. Detection of Microorganisms ASBC) and (Microbiological Control 5. Differential Culture Media ASBC). All the analytical methods listed are validated for their intended uses.

"Total Bacteria" and "Wild Yeast" refer to common beer spoilage organisms that can cause quality issues and often are more resilient to fermentation hurdles than certain foodborne pathogens. "Total Bacteria" refers to lactic acid bacteria, acetic acid bacteria, and other wort- and beer-spoiling bacteria, while "Wild Yeast" refers to yeasts not normally used in brewing or spoiling organisms such as Brettanomyces and non-Saccharomyces yeast.

Lactic acid bacteria (LAB) have a significantly higher heat-resistance in wort and beer when compared to E.Coli O157:H7 and S. typhimurium, and gram-negative LAB/acetic acid bacteria (AAB) and wild yeasts are highly adapted for growth in restrictive fermentation environments (pH < 4.2, alcohol content, hop compounds, yeast fermentation metabolites). These quality indicator organisms, "Total Bacteria" and "Total Wild Yeast," represent undesired microbial growth during fermentation-based ingredient manufacturing that is inhibitive to common foodborne pathogens, and are appropriate indicator organisms for microbiological testing for our manufacturing process and intended use.

Additionally, we would like to state a specification limit for Enterobacteriaceae in OYR-481 as no greater than 10 cfu/g. The microbiological results from a minimum of three non-consecutive batches of OYR-481 demonstrate that the ingredient can be manufactured to meet this specification limit and is included in Table 2 below.

The analytical method used to test for Enterobacteriaceae in OYR-481 has method reference CMMEF Chapter 9.62 and this method is validated for the intended use.

The specification limit for lead in OYR-481 is no greater than 5 ppb. The analytical results from a minimum of three non-consecutive batches of OYR-481 demonstrate that the ingredient can be manufactured to meet this specification limit and is included in Table 2 below. The analytical method used to test for lead in OYR-481 has method reference AOAC 2015.01 (modified) and this method is validated for the intended use. This method has a limit of detection (LOD) of 5 ppb and all samples had values below the method LOD and limit of quantification (LOQ).

If a liquid yeast propagation does not meet the QC specifications, then the batch is rejected. Table 1 contains data for three batches of the OYR-481 liquid yeast.

Table 1. Liquid yeast specifications and data for three nonconsecutive batches of OYR-481

Parameter	Specification Range	LC2251 OYR-481 1/20/2022	LC225B OYR-481 1/30/2024	LC225C OYR-481 1/31/2024
Percent Yeast Solids	>3%	4.36%	4.33%	5.17%
Total Viable Cells	>98%	100%	99%	99%
Total Bacteria	<1 per 2 x 10^6 yeast cells	n.d.	n.d.	n.d.
Total Wild Yeast	<1 per 2 x 10^6 yeast cells	n.d.	n.d.	n.d.
Enterobacteriaceae	< 10 CFU per g	< 10 cfu/g	< 10 cfu/g	< 10 cfu/g
Lead	≤ 5 ppb	< 5.00 ppb	< 5.00 ppb	< 5.00 ppb

n.d. not detected

§ 170.235 Part 3, Dietary Exposure

3.1. INTENDED USE OF OYR-481

Omega Yeast Labs, LLC proposes the use of OYR-481 in brewing to improve beer quality and efficiencies in brewing through the reduction of the common off flavor, diacetyl. OYR-481 with enhanced AldB activity will directly convert alpha-acetolactate produced by the yeast into acetoin, bypassing the spontaneous formation of diacetyl when alpha-acetolactate accumulates in the beer. The resulting diacetyl levels will be in line with a high quality beer produced under conditions to minimize diaceyl. Without the AldB enzyme, brewing yeast reduce diacetyl to acetoin, though this process is often rate limiting and sometimes incomplete process in brewing fermentations. OYR-481 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 (Boekhout, 2014). Bread and alcoholic beverages fermented by *S. cerevisiae* have been at the heart of many ancient civilizations. The use of yeast in fermentation can be dated as far back as ancient Egypt. Today the industrial food and beverage applications of *S. cerevisiae* range from bread, alcoholic beverages, coffee, and chocolate to name a few. The manufacturing of *S. cerevisiae* is a global industry with millions of tons of industrial *S. cerevisiae* manufactured each year.

Diacetyl is widely found in fermented products. It is within the ug/L and sometimes mg/L ranges in fermenting beer, and sensory thresholds can be as low as 20 ug/L in pale lagers. The expression of the *B. brevis* AldB acetolactate decarboxylase bypasses the formation of diacetyl through the direct conversion of the diacetyl precursor alpha-acetolactate to the natural yeast biproduct acetoin. Diacetyl in the resulting finished beer measures in 0-20 ug/L. The exposure to the enzyme itself is minimized through the intracellular expression of AldB. Once the yeast is removed from the beer at the end of fermentation the AldB is also removed. This is of little concern being that it is common practice to add exogenously produced AldB enzyme directly to the fermenting beer. The AldB enzyme is GRAS and a safety evaluation of this enzyme can be found in GRN No. 587.

3.2.2. Estimated consumption

Beer produced with OYR-481 will contain comparable levels of alcohol and flavor metabolites as the host strain, with the exception of reduced levels of diacetyl.

Exposure to the engineered OYR-481 will not differ from the exposure to industrial yeast used in other commercial beer applications. This yeast is flocculant and rapidly declines in viability at the end of fermentation. The settled, non-viable yeast is discarded at the completion of fermentation. The resulting beer contains trace levels of yeast (<3 million cells/ml) and any yeast remaining will have a limited viability and metabolic activity in the packaged beer (Boulton and Quain, 2001).

The following maximum potential exposure estimates for the modified OYR-481 yeast based on potential beer intake for the population of heavy drinkers. It is assumed that only men and women over the age of 21 will be exposed to OYR-481 due to the legal drinking age in the United States.

Assumptions for Calculations:

- Craft Brewery maximum of 1 million cells/ml in the beer product.
- Consumption (Guenther et al., 2013, based on NHANES studies, 2009-2010) of beer for people over the age of 21; Weight (McDowell et al., 2008, National Health Statistics Report).

Heavy Drinking Population	Daily Beer Consumption (ml)	Weight (kg)	Exposure to Yeast (cells/kg bw/day)
Men	1420	88.3	16.1×10^6
Women	1064	74.7	14.3×10^6

AldB exposure:

Based on copy number of Pgk1 (highest level of constitutive expression):

- Pgk1 protein abundance maximum 1,000,000 molecules/cell (Breker at al., 2013)
- 7.4×10^{-14} g/cell of AldB protein
- 74 ng/ml of AldB protein in finished beer

Based on total protein and high range of overexpressed protein:

- Maximum total protein in a yeast cell is 6.0×10^{-12} g/cell (Siwiak and Zielenkiewicz, 2010).
- Maximum 5% of total protein is AldB representing 3×10^{-13} g/cell
- 300 ng/ml of AldB protein in finished beer

Heavy Drinking Population	Daily Beer Consumption (ml)	Weight (kg)	Exposure to AldB (ug/kg bw/day)
Men	1420	88.3	4.8
Women	1064	74.7	4.3

This estimated maximum exposure to OYR-481 does not differ to the exposure to *S. cerevisiae* strains commonly used to ferment beer. The single difference between OYR-481 and the OYL-004 host strain is the targeted insertion of *aldB* gene at the ADE2 locus. The use levels for AldB enzyme (GRN No. 587) corresponds to 50 ADU(L)/L of fermenting wort, which equates to 1.8 ug/ml of the AldB enzyme solids. Thus the AldB enzyme (GRN No. 587) estimate for exposure is 6 times that which is estimated herein for the AldB expressed by OYR-481 with residual yeast (<1 million cells/ml) remaining in finished beer. In addition, commercial brewers will further reduce yeast to undetectable levels through cold conditioning, centrifuging and/or filtering, thereby reducing or eliminating the human exposure to OYR-481.

§ 170.240 Part 4, Self-Limiting Levels of Use

The use of the proposed yeast in brewing is considered to be self-limiting for technological reasons, such as product flavor profile, which could affect consumer acceptance.

§ 170.245 Part 5, Experience Based on Common Use in Food

While there exists an extensive history of the safe consumption of *S. cerevisiae* yeast and yeast products by both humans and animals, and *S. cerevisiae* products have FDA GRAS status, the statutory basis for our conclusion of its GRAS status in the notice is based on scientific procedures and not common use in food.

§ 170.250 Part 6, GRAS Narrative

6.1. SAFETY ASSESSMENT OF THE HOST STRAIN

The host strain, OYL-004 is an industrial brewing strain of *Saccharomyces cerevisiae*. The origin of OYL-004 is an American brewery. OYL-004 is widely used in commercial beer production of American craft beer styles, including modern American hoppy beer styles.

S. cerevisiae is considered GRAS for use in brewing, baking and winemaking industries (21 CFR §172.896). The genome of *S. cerevisiae* has been extensively studied and it has been determined that the yeast is free of known pathogenicity traits. In addition, several genetically modified *S. cerevisiae* strains for use in fermented beverages have received letters of no objection from the US FDA (GRN No. 120, GRN No. 175, GRN No. 350, GRN no. 798 and GRN No. 841).

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 Environment Protection Agency (EPA) has included *S. cerevisiae* as a recipient microorganism that is eligible for exemptions from EPA review under the Toxic Substances Control Act and/or expedited EPA review (40 CFR 725.420). The inclusion of *S. cerevisiae* in the list of recipient microorganisms that are eligible for exemption is based upon the demonstrated lack of adverse effects for this species. 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 is poorly mobilizable.

According to the European Food Safety Agency (EFSA), yeasts used in food production, particularly baker's/brewer's yeast, are considered among the safest of microorganisms (EFSA, 2007; EFSA, 2013) *S. cerevisiae* has been designated Qualified Presumption as Safe (QPS) status in Europe, which indicates that no additional safety assessment is needed according to the established guidelines (EFSA, 2007; EFSA, 2008).

The OYL-004 host strain belongs to the *Saccharomyces cerevisiae* species which has been used for more than 7,000 years by humans in fermented beverages. It can therefore be concluded that species will remain GRAS, even after genetic transformation, as long as the transformation does not introduce new or changed capabilities for harmful effects.

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

6.2.1. Foreign genetic material source and product

Source: *Brevibacillis brevis*

A complete safety evaluation of *B. brevis* is not relevant for this GRAS determination, because *B. brevis* as an intact organism is not part of the construction process of OYR-481. *B. brevis* is rarely associated with infection diseases and is commonly found in soil, air and water. As previously mentioned, the *aldB* gene sequence from *B. brevis* was codon optimized for expression in *S. cerevisiae* and a synthetic gene was used to generate OYR-481, thus negating a possibility for transfer of any other donor DNA.

In conclusion, *B. brevis* is a suitable gene donor for the modified *S. cerevisiae* strain OYR-481 for use in beer production.

Product: The AldB acetolactate decarboxylase

The *B. brevis* AldB enzyme is an evolutionarily conserved alpha-acetolactate decarboxylase enzyme. These alpha-acetolactate enzymes are found in many bacteria and are essential for the metabolism of valine and isoleucine.

Recombinantly expressed *B. brevis* AldB enzyme had undergone extensive safety evaluation as documented in the GRN No. 587, including 13 week oral toxicity studies in rats, a reverse mutagenesis assay (Ames test), and an in vitro micronucleas assay. This recombinantly expressed and purified AldB is widely used in the production of beer and dosed into the fermentation medium. Unlike the yeast-expressed AldB which is removed with the yeast post fermentation, the AldB dosed into beer remains in the finished beer product. Thus the exposure to the AldB expressed by OYR-481, will be significantly less then the commercial AldB enzyme.

The amount of diacetyl measured in beer fermentations with enhanced AldB activity (0-20 ppb) are similar to those found in standard beer fermentations employing methods to reduce diacetyl. Furthermore, the *aldB* gene present in the OYR-481 strain resulting from the genetic modification does not code for either toxic proteins, nor proteins implicated in the formation of undesirable compounds. A literature search performed on Jan 22nd 2024 with the words "aldB AND toxin" and "aldB AND toxic" produced four results on pubmed.gov, none of which were relevant to the *aldB* gene present in OYR-481 (the papers were unrelated or studied the detoxifying effects of aldehyde dehydrogenases [Bergeron et al. 1998, Manow et al. 2020]). Thus, there was no evidence in the literature to indicate toxicity of AldB.

Given the reported specificity of AldB to alpha-acetolactate and the levels of diacetyl measured in beer fermentation, expression of AldB in *S. cerevisiae* brewing yeast can be reasonably considered as safe.

6.2.2. Native genetic material source and product: The promoter and terminator

The promoter and terminator sequences are native to *S. cerevisiae* and are well-characterized, commonly used non-coding regulatory elements. These function to regulate the expression of *aldB* in OYR-481.

6.2.3. Construction of the modified organism

6.2.3.1. Vector and repair template

The *ADE2* disruption and *ADE2* repair CRISPR plasmids (Figure 2) were constructed using standard molecular cloning techniques. A yeast shuttling vector containing Cas9 regulated by *S. cerevisiae PGK1* promoter and terminator was digested with BsmBI. An *ADE2* disruption or repair sgRNA was synthesized as oligonucleotides, annealed, phosphorylated and ligated to the BsmBI yeast shuttling vector containing tRNA phe promoter, tRNA phe and the tSRN52 sequences for optimal expression and stability of the sgRNA. The final CRISPR plasmids were confirmed with Oxford Nanopore full plasmid sequencing.

A repair template for homology-directed repair of the induced double strand break was prepared by PCR from a vector containing the synthesized gBlock. The sequence of the repair template contains the *aldB-ADE2* sequence and homology to regions upstream and downstream of the CRISPR-Cas9 cut site (schematic provided in Figure 3).

6.2.3.2. Transformation of the host strain

The *ADE2* CRISPR plasmids, the ADE2 disruption oligonucleotide, and *aldB-ADE2* repair template were the only DNA sequences used in the transformation method.

OYL-004 was transformed with *ADE2* disruption CRISPR plasmid and *ADE2* disruption oligonucleotide and plated onto YPD-G418 media to select for transformed cells. Single colonies were chosen and re-streaked to YPD-G418 media. These transformants were screened by red colony color and with PCR primers that anneal to the upstream and downstream region of *ade2* disruption scar. Thus, only successful CRISPR events that led to the *ade2* disruption resulted in a 598 bp PCR product. The resulting strain was passaged in liquid YPD media, plated to single colonies and replica plated on YPD-G418 plates. Colonies that no longer displayed G418-resistance were transformed with the *ADE2* repair CRISPR plasmid and *aldB-ADE2* repair template. Initial G418 colonies were restruck to YPD-G418 media and screened for white colony color and with PCR primers that anneal within the aldB cassette and downstream the insertion within the ADE2 orf. Successful ADE2 repair events resulted in a 1166 bp product. The resulting strain was passaged in liquid YPD media, plated to single colonies and replica plated on YPD-G418 plates. The lack of G418-resistance confirmed the loss of the *ADE2* repair CRISPR plasmid (Figure 4).

The results show that the *ADE2* CRISPR plasmids containing the G418 selection marker are not present in OYR-481 and that the CRISPR-Cas9 editing event resulting in the insertion of the aldB expression cassette was specifically targeted to the *ADE2* locus.

6.3. SAFETY ASSESSMENT OF THE MODIFIED ORGANISM

The safety assessment evaluates the exposure to OYR-481, though the presence of OYR-481 in the final beer product is minimal and is further reviewed in section 6.4.2.3.

6.3.1. Characterization of the transformation event

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 δ12 (5'-TCAACAATGGAATCCCAAC-3') and δ21 (5'-CATCTTAACACCGTATATGA-3') to amplify these δ sequences, we have verified the genetic relationship between the host strain OYL-004 and OYR-481 (Figure 6).

Loss of the G418 antibiotic resistance marker was confirmed by streaking OYR-481 onto YPD-G418 media and confirming sensitivity. Figure 4 clearly shows that OYR-481 is sensitive to this antibiotic like the parental strain OYL-004.

Colony PCR of the resulting isolates was performed to confirm the insertion of *aldB* expression cassette at position +600 in the *ADE2* promoter. The presence of the 1166 bp PCR fragment in OYR-481 and absence in the OYR-004 is shown in Figure 5.

6.3.2. Effect of the genetic modification on the physiology of the OYR-481

6.3.2.1. Overview of the function of the *aldB* gene

The *aldB* gene encodes an acetolactate decarboxylase that catalyses the confersion of alpha-acetolactate to acetoin in fermentation. During fermentation alpha-acetolactate is produced by brewing yeast and released into the fermenting beer where it is converted into diacetyl via a spontaneous non-enzymatic decarboxylation reaction. Diacetyl is a common off flavor in beer resulting in a buttery flavor and slick mouthfeel. Brewing yeast naturally uptake the diacetyl at the end of fermentation and convert it to acetoin. The expression of AldB by brewing yeast allows this conversion to happen directly from alpha-acetolactate and bypass the production of the diacetyl off flavor. In beer brewed with OYR-481 diacetyl is maintained below 20 ug/L threshold throughout fermentation.

6.3.2.2. Growth and fermentation rates of OYL-004 and OYR-481 during laboratory-scale fermentations

Brewing trials with OYL-004 and OYR-481 showed similar fermentation rates, degree of fermentation, and terminal pH. Propagation trials with OYL-004 and OYR-481 resulted in similar peak cell densities. No growth advantages were observed in OYR-481 relative to the OYL-004 host strain. All data indicate that the only difference between OYL-004 and OYR-481 is the enhanced expression of *aldB*.

Therefore, it can be concluded that the OYR-481 strain is substantially equivalent to the host strain OYL-004 except for the enhanced expression of *aldB*.

6.3.2.3. Sensory and chemical analysis of beer brewed with OYR-481 and OYL-004

Fermentations were performed for sensory analysis of the OYR-481 and OYL-004 host strain. Wort was prepared from barley malt (15°P extract). Fermentations were carried out at 23°C for 7 days. Descriptive sensory analysis was performed by a panel of 6 trained tasters. All 6 tasters were able to discern between the OYR-481 and OYL-004 parent strain, indicating a significant difference perceivable diacetyl. The samples were analyzed by GC-ECD and diacetyl concentration reached 216 ug/L in the fermentation with OYL-004 relative to 11 ug/L in fermentations with the OYR-481, a 20-fold increase. No other notable differences in aroma profile were observed by sensory.

6.3.3. Allergenic/Toxigenic potential

The *B. brevis aldB* gene present in the OYR-481 strain resulting from the genetic modification does not code for either toxic or allergenic proteins, nor proteins implicated in the formation of undesirable compounds. A literature search performed on January 22nd, 2024 with the words "AldB AND toxin" and "AldB AND toxic" produced four results on pubmed.gov, none of which were relevant to the *aldB* gene present in OYR-481 (the papers were unrelated or studied the detoxifying effects of aldehyde dehydrogenases [Bergeron et al. 1998, Manow et al. 2020]). Thus, there was no evidence in the literature to indicate toxicity of AldB. A bioinformatics comparison of AldB to known allergens was performed on January 22nd, 2024 using a well-documented allergen database (AllergenOnline version 21, http://www.allergenonline.org/) with an 80mer sliding window. Of the 206 80mers in AldB, no matches were found with greater than 35% identity. A bioinformatics search was performed using NCBI BLASTp on January 22nd, 2024 on each of the 19 introduced open reading frames resulting from the insertion of the *aldB* expression cassette at the ADE2 locus. The criteria used for BLASTp were default parameters: BLOSUM92 scoring matrix, Word size 6, Expect value 10, hitlist 100, Gapcosts 11,1, window size 40, threshold 21. No sequence alignments with reported toxins were identified. This analysis supports the conclusion that AldB and the resulting OYR-481 strain do not have any known associated allergen or toxin risks.

In addition, the AldB enzyme will likely be removed from the finished beer with the OYR-481 yeast as it not a secreted protein. If the yeast undergoes autolysis and AldB is released into the beer, it will be denatured and degraded due to proteolysis. If the AldB is consumed, it will be very susceptible to digestion in the gastro-intestinal system, where a wide range of naturally-occurring food enzymes have been shown to be very liable to acid denaturation and proteolysis.

These considerations lead us to conclude that allergenic or toxic risks related to the presence of the synthetically derived *aldB* gene within the OYR-481 strain are negligible.

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

The insertion of the aldB-expression cassette 600 bp upstream of the *ADE2* locus maintains functional *ADE2* gene by evidenced of absence of a red pigment in the OYR-481 strain. The insertion sites and flanking regions were examined for potential open reading frames. A bioinformatics search for 19 potential open reading frames spanning the site of insertion and the inserted DNA was performed using NCBI BLASTp (http://www.ncbi.nlm.nih.gov/BLAST/) on

January 22nd, 2024. The criteria used for blastp were default parameters: BLOSUM92 scoring matrix, Word size 6, Expect value 10, hitlist 100, Gapcosts 11,1, window size 40, threshold 21. Blast results for each of the 19 open reading frames were manually evaluated for any suggested toxicity and a search for the word toxin was performed on each list of subjects identified. No subjects of these blast searches were known toxins, leading us to conclude that the insertion of the *aldB* expression cassette does not raise any oral toxicity concerns.

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

6.4.1. Changes in brewing procedures as a result of OYR-481 fermentation

The use of the OYR-481 yeast strain leads to no significant changes in brewing procedures, as outlined in Section 6.3.2.3

6.4.2. Changes in beer composition as a consequence of OYR-481 fermentation

6.4.2.1. Global Characteristics of beer

The OYR-481 fermentation results in the off flavor diacetyl remaining below sensory threshold throughout beer fermentaitons. Residual extract, pH, and additional sensory descriptors remain consistent to the OYL-004 host strain. Thus, OYR-481 fermentation will have limited impact on the global beer composition.

6.4.2.2. Flavor modification

Laboratory and pilot scale brewing trials showed that the use of OYR-481 beer yeast strain led to a clean fermentation profile. Descriptive characters of the OYR-481 beer were similar to high quality beers produced the OYL-004 strain employing best practices to reduce diacetyl.

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 OYL-004 and OYR-481 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.

6.4.2.4. Formation of unwanted substances in beer

S. cerevisiae is considered GRAS for use in brewing, baking and winemaking industries (21 CFR §172.896) and the introduced material has been reviewed for known toxin and allergen sequences. Moreover, to the best of our knowledge, the use of the OYR-481 strain in brewing will not lead to the release or the enhancement of undesirable compounds in beer.

6.5. BASIS FOR THE GRAS DETERMINATION

6.5.1. Introduction

The regulatory framework for determining whether a substance can be considered GRAS in accordance with section 201(s) (21 U.S.C. § 321(s)) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. § 301 et. Seq.) ("the Act"), is set forth at 21 CFR 170.30, which states:

> General recognition of safety may be based only on the view of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. The basis of such views may be either (1) scientific procedures or (2) in the case of a substance used in food prior to January 1, 1958, through experience based on common use in food. General recognition of safety requires common knowledge about the substance throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food.
>
> General recognition of safety based upon scientific procedures shall require the same quantity and quality of scientific evidence as is required to obtain approval of a food additive regulation for the ingredient. General recognition of safety through scientific procedures shall ordinarily be based upon published studies, which may be corroborated by unpublished studies and other data and information.

These criteria are applied in the analysis below to determine whether the use of the modified *S. cerevisiae* yeast ingredient in brewing that is the subject of this GRAS determination is GRAS based on scientific procedures. All data relied upon in this GRAS determination are publicly available and generally known, and therefore meet the "general recognition" standard under the Federal Food, Drug, and Cosmetics Act.

6.5.2. Safety Determination

The proposed Omega Yeast Labs brewing ingredient (from *S. cerevisiae*) that is the subject of the current GRAS determination is proposed to be added at approximately 1 million cells per milliliter of wort per degree Plato for brewing wort fermentation, as is standard industrial practice. The proposed yeast ingredient is similar to many other yeast products using *S. cerevisiae* and all have a long history of safe consumption by humans as well as animals. Humans have consumed *S. cerevisiae* through the consumption of its fermentative products, which include bread, beer, and wine.

S. cerevisiae is widely used in industrial fermentation of many food products. It is in the air that we breathe, and it grows naturally on many foods that we eat regularly. It is likely that most humans are exposed daily to live *S. cerevisiae* through consumption of wine, beer, fruits, salads, and cheese and other dairy foods, as well as other traditional and ethnic fermented foods that contain the yeast. This yeast species has been used in food production for over five thousand years, with common industrial applications including baked goods, beer, wine, cider, sake, spirits, coffee and chocolate (Boekhout and Robert, 2014).

Brewer's yeast serves as a model organism for scientific research. Over 30 years of yeast research has contributed to our fundamental understanding of cell and molecular biology. The *S. cerevisiae* genome size is small, is highly homologous with the human genome, genetics are very tractable, and DNA repair pathways are robust allowing for ease of accurate genetic manipulation (Botstein and Fink, 2011). A long history of research and industrial use has indicated that *S. cerevisiae* is safe for use in food and beverage manufacturing (Boekhout and Robert, 2014).

There are over 150 *S. cerevisiae* strains that are commercially sold as brewing yeast. These strains have been isolated from the brewery and farmhouse origins, where the re-pitching or backslopping practices allowed for the propagation and selection of unique brewing characteristics (Gallone et al., 2016). The origin of the host strain, OYL-004, traces to an American Brewery. OYL-004 is a popular strain used by many US craft breweries for traditional English styles and modern American hoppy styles.

The FDA considers *S. Cerevisiae* and several derived products safe for consumption. Indeed, FDA has approved dried yeast as an ingredient for food (21 C.F.R. §172.896), and Baker's yeast extract has been affirmed by the FDA as a GRAS flavoring agent and adjuvant (21 C.F.R. §184.1983). The FDA has also approved various yeast-derived products for their use in food. These include Baker's yeast protein (21 C.F.R. §172.325), Yeast-malt sprout extract (21 C.F.R. §172.590) and Baker's yeast glycan (21C.F.R. §172.898). *S. cerevisiae* is also considered Generally Recognized as Safe as noted in several GRAS Notices; GRNs 120, 175, 239, 260, 284, 350, 353.

S. cerevisiae has a long history of being considered non-pathogenic. Regulatory authorities have evaluated the safety of *S. cerevisiae* and consider the organism safe for use in food manufacturing.

Several GRAS notifications for modified strains *of S. cerevisiae* used in winemaking have been sent to FDA, and the agency has responded that they had no further questions on the GRAS determinations. In summary, regulatory authorities have reviewed the extensive safety database on *S. cerevisiae* and found no issues of concern with respect to its use in human food or human food production.

6.5.3. General recognition of the safety of a modified *Saccharomyces cerevisiae* for use in brewing

The intended use of the modified *S. cerevisiae* has been determined to be safe through scientific procedures, as set forth in 21 CFR § 170.3(b), thus satisfying the so-called "technical" element of the GRAS determination, and this determination is based on the following:

- The Omega Yeast Labs modified yeast ingredient (OYR-481 strain) that is the subject of the current GRAS determination is derived from *S. cerevisiae*, also (also known as "bakers" or "brewers" yeast).

- The OYR-481 strain was developed by Omega Yeast Labs to reduce levels of the off flavor diacetyl, by enhancing the activity and expression of *B. brevis aldB*. *S. cerevisiae* produces many aroma-active metabolites that contribute to the complexity of a beer's flavor including phenolics, esters, carbonyl compounds, higher alcohols, aldehydes and sulfur compounds. Some of these metabolites are highly sought after for tropical fruit flavors. This strain was modified with CRISPR-Cas9 introducing the insertion of codon-optimized DNA sequences that encode for the *aldB* gene, based on the gene native to *B. brevis*. In the modified strain, *aldB* is under the regulation of native promoter and terminator sequences (from *S. cerevisiae*) to promote expression in beer fermentations.

- The OYR-481 yeast strain is intended for use as a commercial liquid yeast culture for the production of fermented beverages in accordance with Good Manufacturing Practices (GMPs). This liquid yeast strain performs alcoholic fermentation in the same manner as its host strain and other traditional brewing strains. The enhanced AldB activity of OYR-481 provides a well-suited brewing yeast strain for American craft beers, hoppy beers and other "clean" beer styles.

- The proposed Omega Yeast Labs OYR-481 (from *S. cerevisiae*) is proposed to be added at approximately 1 million cells per milliliter of wort per degree Plato for brewing wort fermentation, as is standard industrial practice.

- Beer produced with OYR-481 will contain comparable levels of alcohol and flavor metabolites as the host strain, with reduced levels of diacetyl. The engineered OYR-481 will not differ from the exposure to industrial yeast used in other commercial beer applications. This yeast is flocculant and rapidly declines in viability at the end of fermentation. The settled, non-viable yeast is discarded at the completion of fermentation. The resulting beer contains trace levels of yeast (<3 million cells/ml) and any yeast remaining will have a limited viability and metabolic activity in the packaged beer.

- The current US daily intake of yeast in the diet for the US population 2 years and older is as follows: the per-capita average daily intake for yeast from all foods was 1.3 g/day (2.9 g/day, 90th percentile), and the per-user average daily intake was 1.5 g/day (3 g/day, 90th percentile) (FDA, 2021).

- *S. cerevisiae* has a long history of being considered non-pathogenic. It is used in many food manufacturing processes, including bread making, beer brewing, and grape fermentation for wine. The FDA, EPA, and EFSA have evaluated the safety of *S. cerevisiae* and consider the organism safe for use in food manufacturing. Several GRAS notifications for modified strains of *S. cerevisiae* used in winemaking have been sent to FDA, and the agency has responded that they had no further questions on the GRAS determinations.

- The body of publicly available scientific literature on the consumption and safety of *S. cerevisiae* is sufficient to support the safety and GRAS determination of the proposed yeast ingredient. Because this safety evaluation was based on generally available and widely accepted data and information, it also satisfies the so-called "common knowledge" element of a GRAS determination.

- The AldB enzyme from *B. brevis* is GRAS (GRN No. 587) and is widely used in beer manufacturing in the form of a recombinantly expressed and purified enzyme. Beers commercially produced with the direct addition of the AldB enzyme result in reduced diacetyl and are otherwise indistinguishable by flavor and composition to beer produced without AldB enzyme. The expression of the AldB enzyme in brewing yeast provides the same results with the execption of minimal AldB enzyme remanining in the finished beer.

Determination of the safety and GRAS status of OYR-481 that is the subject of this evaluation has been made by Omega Yeast Labs. Omega Yeast Labs has critically reviewed and evaluated the publicly available information summarized in this document and has concluded that the yeast ingredient, produced in a manner consistent with cGMP and meeting the specifications described herein, is safe under its intended conditions of use.

Omega Yeast Labs further concludes that use of OYR-481 in brewing described herein is GRAS based on scientific procedures, and that other experts qualified to assess the safety of food and food ingredients for human consumption would concur with these conclusions.

It is also Omega Yeast Labs' opinion that other qualified scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion. Omega Yeast Labs has concluded that OYR-481 is GRAS under the intended conditions of use on the basis of scientific procedures; and therefore, it is excluded from the definition of a food additive and may be marketed and sold for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21 of the CFR.

Omega Yeast Labs is not aware of any information that would be inconsistent with a finding that the proposed use of the yeast ingredient in brewing, meeting appropriate specifications, and used according to cGMP, is GRAS. Recent reviews of the scientific literature revealed no potential adverse health concerns.

§ 170.250 Part 7, Supporting Data and Information

The following references are all generally available, unless otherwise noted.

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GRNs 120, 175, 239, 260, 284, 350, 353, 587, 798, 841, 928
GRNs 937, 324, 378, 685, 722, 847, 946, 953
FDA. 2021. GRAS Notification: For the use of dried *Saccharomyces cerevisiae* yeast fermentate. Prepared by Cargill, Inc., May 14.

Form Approved: OMB No. 0910-0342; Expiration Date: 07/31/2022
(See last page for OMB Statement)

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

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

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)*: ________

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	Position or Title
Lance Shaner	Owner

Organization *(if applicable)*
Omega Yeast Labs, LLC

Mailing Address *(number and street)*
4720 W Pensacola Ave

City	State or Province	Zip Code/Postal Code	Country
Chicago	Illinois	60641	United States of America

Telephone Number	Fax Number	E-Mail Address
(773) 657-3438		lance@omegayeast.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

Feb 16 2024

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

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

Saccharomyces cerevisiae strain OYR-481

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

☐ Electronic Submission Gateway

☒ Paper

☐ Electronic files on physical media

If applicable give number and type of physical media

3. For paper submissions only:

Number of volumes 1

Total number of pages 37

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

☐ 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) and 170.250(d) and (e))

☐ 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.

Omega Yeast Labs, LLC proposes the use of OYR-481 in brewing to improve beer quality and efficiencies in brewing through the reduction of the common off flavor, diacetyl. OYR-481 with enhanced AldB activity will directly convert alpha-acetolactate produced by the yeast into acetoin, bypassing the spontaneous formation of diacetyl when alpha-acetolactate accumulates in the beer. The resulting diacetyl levels will be in line with a high quality beer produced under conditions to minimize diaceyl. OYR-481 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.

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 of attachments?

☐ Yes ☐ No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Omega Yeast Labs, LLC
(name of notifier)

has concluded that the intended use*(s)* of Saccharomyces cerevisiae strain OYR-481
(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. Omega Yeast Labs, LLC *(name of notifier)* 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.

4720 W Pensacola Ave., Chicago, IL 60641
(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)
Lance Shaner Digitally signed by Lance Shaner Date: 2024.02.15 14:38:33 -06'00'	Lance Shaner	2/15/2024

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)
	OYR-481GRAS Cover Letter	1
	GRAS Determination for Saccharomyces cerevisiae strain OYR-481	2-37

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.

Questions/Comments for GRN 001190

1. Please confirm that the intended use of S. cerevisiae "OYR-481" will be substitutional for the use of other S. cerevisiae strains used in commercial beer production and that its intended use is not expected to increase the dietary exposure to S. cerevisiae.

Yes, the OYR-481 is a substitute for other *S. cerevisiae* strains. There will not be an increase in dietary exposure in use cases of OYR-481 as compared to *S. cerevisiae*.

2. On page 22, the notifier calculated the amount of acetolactate decarboxylase (AldB) per mL of the finished beer based on the copy number of Pgk1 or the total protein.
(a) Please specify what Pgk1 stands for and explain how 7.4 x 10-14 g/cell of AldB was calculated.

Pgk1 is the yeast 3-phosphoglycerate kinase, a key enzyme in glycolysis and glucogenesis. It is estimated to be one of the most abundant proteins in the cell at 535389 +/- 420545 molecules/cell (Breker et. al 2013). We used 1,000,000 molecules per cell as a high estimate for Pgk1 abundance representing a theoretical example of a highly expressed protein in the cell. The molecular weight of AldB is 31.58 kDa, which if you divide by Avogadro's number (6.022e+23) is 5.24e-20 grams per molecule. At 1,000,000 molecules per cell, this would equate to 5.24e-14 grams per cell. (Acknowledgement in an error where 7.4 x 10-14 g/cell was calculated from the molecular weight of Pgk1 which is 44.7 kDa rather than the AldB molecular weight of 31.58 kDa).

(b) Please clarify what the basis is for the assumption that the maximum 5% of total protein in a yeast cell is AldB.

The limit to the overexpression of a specific protein in a yeast cell has been estimated to be 15% of the total protein (Eguchi et al. 2018). This limit was established for multiple copy number plasmid-based expression (100+ copies) under a strong constitutive promoter. With a low copy number under a strong constitutive promoter, such as a gene integrated into the genome (4 copies in the OYR-481 tetraploid strain), the protein level is estimated to be 1-5%. Therefore 5% was used as our basis for a highly expressed protein from a genomic locus, as is the case for the AldB gene in OYR-481.

(c) Please provide full citations for Breker et al., 2013 and Siwiak and Zielenkiewicz, 2010 that are missing in Part 7 of GRN 001190.

Breker, M., Gymrek, M., & Schuldiner, M. (2013). A novel single-cell screening platform reveals proteome plasticity during yeast stress responses. The Journal of cell biology, 200(6), 839–850. https://doi.org/10.1083/jcb.201301120

Siwiak, M., & Zielenkiewicz, P. (2010). A comprehensive, quantitative, and genome-wide model of translation. PLoS computational biology, 6(7), e1000865. https://doi.org/10.1371/journal.pcbi.1000865

Eguchi, Y., Makanae, K., Hasunuma, T., Ishibashi, Y., Kito, K., & Moriya, H. (2018). Estimating the protein burden limit of yeast cells by measuring the expression limits of glycolytic proteins. eLife, 7, e34595. https://doi.org/10.7554/eLife.34595

3. For the strain identity, please state whether S. cerevisiae "OYR-481" has been deposited in a recognized culture collection and provide the deposit designation. If it has not been deposited, please describe how the parental strain "OYL-004" is taxonomically classified.

The *Saccharomyces cerevisiae* parent strain "OYL-004" originates from the BRY-96 "Beer Pitching Strain" and is deposited as NRRL Y-7407 and NCYC. The designation of "OYL-004" is a tradename and is synonymous with BRY-96, WLP001 and WY1056. The *S. cerevisiae* taxonomy has also confirmed with whole genome sequencing (accession numbers: OYL-004 SAMN15364566, BRY-96 SAMN15364561, WLP001 SAMN15364540, WY1056 SAMN15364542).

4. For the administrative record, please clarify whether genome data of S. cerevisiae "OYR-481" is available in the public domain, e.g., a NCBI accession number.

The "OYL-004" parent strain genome data is available under the NCBI accession number: SAMN15364566. The modified "OYR-481" genome data is not publicly available.

5. Please confirm that S. cerevisiae "OYR-481" is non-pathogenic and non-toxigenic by briefly summarizing its genotypic or phenotypic characteristics.

The OYR-481 exhibits genotypic and phenotypic characteristics consistent with the *S. cerevisiae* host strain. OYR-481 grows in aerobic and anaerobic conditions and is Crabtree positive, which makes it well suited for alcoholic fermentations. It ferments mono, di and tri-saccharide sugars and produces desired fermentation products such as carbon dioxide, ethanol, esters and organic acids. The OYR-481 strain exhibits a strong flocculation phenotype which allows the efficient separation from beer after fermentation is complete. There are no known toxins or virulence factors produced by *S. cerevisiae* or the resulting OYR-481 strain that would lead to pathogenic or toxigenic effects. Humans have been consuming and producing fermented products with *S. cerevisiae* as far back as we can trace, demonstrating *S. cerevisiae* is GRAS on the basis of historic use.

6. For the administrative record, please clarify how the purity of the S. cerevisiae "OYR-481" inoculum for the manufacturing process is ensured.

The “OYR-481” inoculum is stored as a glycerol stock at -80°C. This inoculum is the original monoclonal isolate that is described in Part 2 of the prepared GRAS dossier and the purity of this inoculum has been confirmed by interdelta PCR. The manufacturing process starts from the glycerol stock, which is struck to YPD agar media at 20°C for 3 days and stored at 4-10°C for a maximum of 30 days. The manufacturing process as described in Part 2.5 of the prepared GRAS dossier is monitored continuously through standard microbiological plating and PCR at each stage to confirm purity.

7. The notifier states that “OYR-481 yeast strain is intended for use as a commercial liquid yeast culture for the production of fermented beverages in accordance with Good Manufacturing Practices (GMPs).” Please also confirm that the S. cerevisiae “OYR-481” manufacturer implements GMPs during the strain production.

Yes, that is correct. The manufacturer adheres to GMPs throughout the entire production process of the “OYR-481” strain.

8. Please clarify that the fermentation process is continuously monitored for contaminants.

Yes, that is correct. The fermentation process for “OYR-481” is continuously monitored for contaminants throughout each phase outlined in Part 2.5 of the dossier.

9. To support the safety conclusion, the notifier lists the use of genetically modified S. cerevisiae strains in fermented beverages that have received No-Questions Letter from FDA (GRNs 000120, 000175, 000350, 000798 and 000841). We note that GRNs 001062 and 001096 also received a No-Questions Letter from us in 2023. As each GRAS notice stands on its own, for the administrative record, please provide a brief paragraph for each of these GRNs summarizing the information pertaining to safety for this GRAS notice.

GRN 000120 – The “ML01” strain is similarly a modified *S. cerevisiae* strain used in the production of alcoholic beverages. The ML01 strain includes enzymes to catalyze the conversion of L-malate to L-lactate in wine fermentations, avoiding a secondary malolactic fermentation process with lactic acid bacteria. Both the ML01 strain and OYR-481 were modified using genetic engineering technology.

GRN 000175 – The “ECMo01” strain is similarly a modified *S. cerevisiae* strain used in the production of alcoholic beverages. The ECMo01 strain enhances the expression of yeast enzymes that reduce amounts of the undesirable ethyl carbamate compound produced from urea in wine fermentations. Both the ECMo01 strain and OYR-481 were modified using genetic engineering technology.

GRN 000350 – The “P1Y0” strain is similarly a modified *S. cerevisiae* strain used in the production of alcoholic beverages with reduced off flavors, specifically P1Y0 leads to the reduction of hydrogen sulfide in wine fermentations. Both the P1Y0 strain and OYR-481 were modified using genetic engineering technology.

GRN 000798 – The “yBBS002” strain is similarly a modified *S. cerevisiae* brewing strain used in beer fermentations to improve the overall aroma properties of beer. In this case, the yBBS002 strain produces the linalool and geraniol hop compounds imparting overall hoppy character. Both yBBS002 and OYR-481 were modified using CRISPR/cas9 gene editing technology.

GRN 000841 – The “Saccharomyces cerevisiae expressing L-lactate dehydrogenase from Rhizopus oryzae” strain is similarly a modified *S. cerevisiae* brewing strain used in beer fermentations to introduce an enzymatic activity replacing the use of another food additive. In this case, the enzyme activity is L-lactate dehydrogenase avoiding the need for a lactobacillus souring step. Both the LDH expressing strain in GRN 000481 and OYR-481 were modified using genetic engineering technology.

GRN 001062 – The “OYR-185” strain is similarly a modified *S. cerevisiae* brewing strain used in beer fermentations to improve the overall aroma properties of beer. Both OYR-243 and OYR-481 were modified using CRISPR/cas9 gene editing technology.

GRN 001096 – The “OYR-243” strain is similarly a modified *S. cerevisiae* brewing strain used in beer fermentations to improve the overall aroma properties of beer. Both OYR-243 and OYR-481 were modified using CRISPR/cas9 gene editing technology.

10. Please provide updated information on the literature search(es) performed to prepare the notice. This includes the date(s) (e.g., month and year) of the search(es), the resource database(s) used (e.g., PubMed), the principal search terms used, and the time period that the search spanned (e.g., 1/2000 to 1/2024).

Literature searches for the prepared notice were conducted using PubMed from 7/2023 to 1/2024 and references are noted throughout. Specific searches for estimates of dietary exposure on Saccharomyces Genome Database (yeastgenome.org) using “PGK1” were performed from 07/2023 to 01/2024. Specific searches on for the toxigenic potential of AldB were performed on Pubmed on Jan 22nd 2024 using search terms “aldB AND toxin” and “aldb AND toxic” are referenced within Part 7.