GRN 841

GRAS Conclusion

Saccharomyces cerevisiae with lactate dehydrogenase from *Rhizopus oryzae* is Generally Recognized As Safe for Use in the Manufacture of Beer

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1. Signed Statements and Certifications

1.1 Exemption from Premarket Approval

Mascoma LLC has determined that its *Saccharomyces cerevisiae* expressing a gene encoding a sequence of lactate dehydrogenase from *Rhizopus oryzae* is a Generally Recognized as Safe ("GRAS") substance for the intended food application and is, therefore, exempt from the requirement for premarket approval from the Federal Food, Drug and Cosmetic Act.

1.2 Basis for GRAS Determination

The determination of the GRAS status is based on scientific procedures and conforms to the regulations in accordance with 21 CFR § 170.30(a) and (b).

1.3 Name and Address of Notifier

Mascoma LLC
67 Etna Road, Suite 200
Lebanon, New Hampshire, 03766

1.4 Common Name of the Notified Substance

Saccharomyces cerevisiae expressing a gene encoding a lactate dehydrogenase from *Rhizopus oryzae*.

1.5 Intended Conditions of Use

The modified *Saccharomyces cerevisiae* encoding for the wild-type lactate dehydrogenase from *Rhizopus oryzae* is GRAS when used in the production of alcoholic beer. The modified yeast product is produced by fermentation and is intended for use in beer fermentation to produce alcohol and impart a sour flavor. Typically in the commercial production of alcoholic beers, the product is pasteurized and filtered, which inactivates and removes the yeast in the final product. However, in some craft breweries, the yeast is not completely removed from the initial fermentation and the remaining yeast may be used in a secondary fermentation, although normally a yeast specific for re-fermentation is used, making it less likely that the modified yeast will remain in the product. For this application, the modified *Saccharomyces cerevisiae* is intended to replace yeast from other available commercial sources in the fermentation of beer.

1.6 Availability of Information for FDA Review

A notification package providing the information that supports this GRAS conclusion is enclosed. The package includes a safety evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure. The complete data and information that are the basis for this GRAS conclusion are available for review and copying at 67 Etna Rd, Suite 200, Lebanon, NH 03766.

1.7 Disclosure and Certification

Parts 2 through 7 of this notification do not contain any data and or information that is exempt from disclosure under the Freedom of Information Act.

Mascoma LLC certifies to the best of our knowledge that this GRAS notice is complete, representative and balanced and includes unfavorable information as well as favorable information known to us and pertinent to the evaluation of the safety and GRAS status of the use of the notified substance.

Signature of Authorized Official

Joanne Donoghue
Director, EHS & Operations
Mascoma LLC

2. Identity, Method of Manufacture, Specifications, and Technical Effect

2.1 Identity of Notified Substance - Enzyme

IUB Name:	L-lactate dehydrogenase
Other name(s):	Lactic acid dehydrogenase; L(+)-nLDH; L-(+)-lactate dehydrogenase; L-lactic dehydrogenase; L-lactic acid dehydrogenase; lactate dehydrogenase; lactate dehydrogenase NAD-dependent; lactic dehydrogenase; NAD-lactate dehydrogenase
Systematic name:	(*S*)-lactate:NAD^+ oxidoreductase
IUBMB No.:	1.1.1.27
CAS No.:	9001-60-9
Reaction:	(*S*)-lactate + NAD^+ = pyruvate + NADH + H^+; Also oxidizes other (*S*)-2-hydroxymonocarboxylic acids. $NADP^+$ also acts, more slowly, with the animal, but not the bacterial, enzyme.
Amino acid sequence:	The amino acid sequence is provided in **Appendix 1**.

L-lactate dehydrogenase, EC 1.1.1.27, is classified as an oxidoreductase with the alcohol group of the lactate molecule as the hydrogen donor and NAD+ as the hydrogen acceptor (EC number 1.1.1) and is the 27th enzyme to be categorized within this enzyme group.

LDH is ubiquitous in nature. The enzyme is found in all five kingdoms and, thus, includes plants, animals and microorganisms. Human LDH has been reported in the literature by Ringoir and Plum (1975), Emes (1974), Markel and Janich (1974), McQueen (1974), Burd and Usatequi-Gomez (1973), and McKee *et al.* (1972); that from pig by Eventoff *et al.* (1974). Adams *et al.* (1973) and Taylor *et al.* (1973) have reported on dogfish LDH, and Carlotti *et al.* (1974) and Ryan and Vestling (1974) on that of rat liver and hepatomas. Fritz *et al.* (1973) reported on different rates of tissue turnover of the rat isozymes. Kabara and Knovich (1972) extracted LDH isozymes from mouse brain. Ehmann and Hultin (1973) studied chicken breast LDH M5. Eby *et al.* (1973) reported on frog LDH and Lim *et al.* (1975) on that from salmonid fish. Long and Kaplan (1973) reported on horseshoe crab and sea worm LDH. Rothe (1974) studied LDH in potatoes and Kato-Noguchi isolated LDH from fresh-cut carrots (1998). Brown *et al.* (1975) and Allsopp and Matthews (1975) reported on the Actinomyces and Mycoplasma enzymes. Skory published the first report of fungal LDH expressed in yeast. L-LDH-A derived from *Rhizopus oryzae* that was expressed in *S. cerevisiae* (2003).

LDH is an important enzyme in humans and is found in virtually all cells, and is found in many body tissues, especially the heart, liver, kidney, muscles, blood cells, and lungs. Adeva-Andany, *et al.* provide a comprehensive review on lactate metabolism in humans. Lactate is a

hydrocarboxylic acid that may exist in the human body as two stereoisomers, L-lactate and D-lacate; L-lactate is the predominant physiological enantiomer. As the pKa of the pair lactate/lactic acid is 3.8, the anion lactate is the predominant moiety that appears in the human body. Analogous to lactic acid, pyruvic acid is a strong organic acid existing as anion pyruvate at human body pH values. L-lactate is either produced or removed by a reversible oxido-reduction reaction catalyzed by the enzyme L-lactate dehydrogenase (LDH), which is principally located to the cytosol of human cells.

In one direction of the reaction, pyruvate is reduced to produce L-lactate while reduced nicotinamide adenine dinucleotide (NADH) is oxidized to nicotinamide adenine dinucleotide(NAD+).This reaction is thermodynamically favored. In the opposite direction, L-lactate is oxidized to form pyruvate whereas NAD+ is reduced to NADH (Le *et al.*, 2010; Adeva-Andany *et al.*, 2014).

Isozymes of LDH occur in different regions of the body, each region having a unique conformation of different subunits. The current literature proposes that there are at least six LHD isozymes (Augoff *et al.*, 2015). LDH is a tetrameric enzyme composed of two protein subunits which total approximately 135 kDa (Cahn, 1962). LDH has many isoforms, which are all tetramers of two different kinds of subunit: the H (from heart) subunit, or the M (muscle) subunit. It is the combination of these subunits which give a specific LDH its properties.

The tetramer can assemble as five separate isozymes by forming all combinations of the M (muscle) form (product of the Ldh-A gene) or the H (heart) form (product of the LDH-B gene) producing: M_4 (= A_4 = LDH5), M_3H_1 (= A_3B_1 = LDH4), M_2H_2 (= A_2B_2 = LDH3), M_1H_3 (= A_1B_3 = LDH2), and H_4 (= B_4 = LDH1) (Rogatzki, 2015). LDH-A, LDH-B and LDH-C are L isomers, whereas LDH-D is a D isomer. The L isomers use or produce L-lactate, which is the major enantiomer found in vertebrates (Valvona, 2016).

LHD is a protein that helps produce energy in the body. LDH is often measured in humans to check for tissue damage. The normal value range is 105-333 international units per liter (IU/L), and values may vary from lab to lab based on test methodology and other testing variation. https://medlineplus.gov/ency/article/003471.htm). LDH is released from stressed or damaged cells. The literature reports that values can vary with age, especially during periods of growth in children. LDH isozymes in the developing fetus have been reported on by Werthamer et al. (1973) and their variations with age by Fegeler and Gerlach (1972). See also Ringoir and Plum (1975), Mitsutaka (1974), Glass and Doyle (1972), and Wilkinson and Walter (1972). Still, a higher-than-normal level may indicate various health issues, including ischemia, heart attack, muscle injury, and cancer. Thus, LDH is of interest clinically in that the serum level of certain isozymes reflects pathological condition in particular tissues. In one study, salivary glands measurements from control subjects: 134.7 IU/L in men and 54.5 IU/L in women, with higher values in oral cancer (men: 580.6, women: 418.4) and oral submucosal fibrosis patients (men: 398.6, women: 727.2) (Vasavi kadiyala, 2015).

Structures of LDH from a range of species are well represented in the Protein DataBank. A high level of structural similarity is apparent despite significant differences in primary structure between each of these enzymes (Pineda *et al.*, 2007).

2.2 LDH and Lactate

The study of lactate metabolism dates as far back as the 1700s when it was first isolated in 1780 by Karl Wilhelm Scheele, followed by research conducted by Jöns Jacob Berzelius about 1815 that proposed the significance of lactate metabolism. Berzelius was the first to observe that lactate accumulated following intensive activity. However, lactate was misunderstood as it was seen as "passive poisoning" of the body bringing on exhaustion. Thus, lactate's role was not seen as a constructive one and, for the next 200 years, it was largely considered a waste product. Lactate is important to understanding and appreciating the significance of LDH.

Lactate dehydrogenase, LDH, is the key enzyme in lactate production. It can make pyruvate from lactate, or lactate from pyruvate, with the concomitant production of NADH or NAD+. And when concentrations of lactate are high – as in intense exercise for instance – the excess lactate creates a negative feedback on LDH, thereby decreasing its activity.

LDH is a key enzyme in anaerobic respiration. Anaerobic respiration is the conversion of pyruvate into lactic acid in the absence oxygen. This pathway is important to glycolysis in two main ways. The first is that if pyruvate were to build up, glycolysis and thus the generation of ATP would slow. The second is anaerobic respiration allows for the regeneration of NAD+ from NADH. NAD+ is required when glyceraldehyde-3-phosphate dehydrogenase oxidizes glyceraldehyde-3-phosphate in glycolysis, which generates NADH. Lactate dehydrogenase is responsible for the anaerobic conversion of NADH to NAD+.

$$\text{Pyruvate}^- + \text{NADH} + \text{H}^+ \leftrightarrow \text{Lactate}^- + \text{NAD}^+$$

The literature reports that when plant cells lack oxygen and oxidative phosphorylation is blocked, NADH formed in the course of glycolysis is initially oxidized with the participation of LDH, and dissociation of the produced lactic acid induces acidification of the cytoplasm. A decrease in pH inhibits LDH and activates other enzymes, such as pyruvate decarboxylase, which directs the metabolic pathway from lactic acid to ethanol. Any failure to switch the metabolic pathways results in the accumulation of lactic acid causing the cytoplasmic pH to drop further, which may lead to cell death (Kulichikhin *et al.*, 2009). The yeast *Candida sonorensis* has been reported to express both bacterial and fungal lactate dehydrogenases to produce L-lactic acid (Ilmén *et al.*, 2013). *Aspergillus niger* is another organism that expresses LDH for the production of lactic acid (Dave & Punekar, 2015). The literature reports that *Rhizopus orzyae* ATCC 9373 is a lactic-acid producing strain (Büyükkileci *et al.*, 2006). *Aspergillus brasiliensis* is used to overexpress the LDH-A gene from *Rhizopus oryzae* (Liaud *et al.*, 2015).

2.2.1 LDH from Rhizopus oryzae

The lactate dehydrogenase gene encoded in the *S. cerevisiae* was amplified by PCR from an artificially synthesized gene based on the Genbank sequence.

Table 1. Source of the Introduced Gene

Gene	Enzyme	EC/TC number	Donor Organism	Genbank Accession No.	Source of inserted genetic material
Lactate dehydrogenase (ldhA)	LDH	1.1.1.27	*Rhizopus oryzae*	ABL84845	Synthesized and codon optimized for *S. cerevisiae*

Information on the source of the inserted genetic material is provided in **Table 1**.

2.3 Production Organism and Construction

2.3.1 Production Strain

The production organism is a strain of *Saccharomyces cerevisiae* that is encoded with a lactate dehydrogenase gene that is native to *Rhizopus oryzae*. The gene was amplified by PCR from an artificially synthesized gene based on the Genbank sequence, which negates the possibility of donor DNA transfer to the strain.

The genetically modified production organism complies with OECD (Organization for Economic Cooperation) and criteria for GILSP (Good Industrial Large Scale Practice) microorganisms and meets the criteria for a safe production microorganism as described by various experts (Pariza & Foster, 1983; IFBC, 1990; OECD, 1993; Pariza & Johnson, 2001; JECFA 2001, 2006).

The production strain has been confirmed via genetic sequencing to belong to the genus *Saccharomyces* using the large subunit ribosomal rRNA (LSU) region as a marker for genus identification, as these regions are highly conserved. For species identification, the divergence regions of this rDNA LSU, D1 and D2, were further compared to confirm the species as *cerevisiae*. In addition, whole genome sequencing was completed for the strain.

Taxonomic characteristics of the parent yeast:

Name:	*Saccharomyces cerevisiae*
Class:	*Saccharomyces*
Order:	*Saccharomycetales*
Genus:	*Saccharomyces*
Species:	*cerevisiae*

In addition to other commonly used names associated with *Saccharomyces cerevisiae* (e.g. yeast, baker's yeast, brewer's yeast, and lager beer yeast), the taxonomic literature lists other synonyms such as *Saccharomyces bayanus*, *Saccharomyces carlsbergensis*, *Saccharomyces uvarum*, *Saccharomyces sake*, and *Saccharomyces vini* because the classification has undergone many changes over the years (Lodder & Kreger-van-Rij, 1952; Lodder, 1970; Demain *et al.*, 1998; Barnett *et al.*, 1983). Lodolo *et al.* provides a review of *Saccharomyces cerevisiae* in beer brewing (2008)

The production *Saccharomyces cerevisiae* strain has been genetically modified to express a lactate dehydrogenase enzyme which catalyzes the reversible reaction of pyruvate to lactate. In an anaerobic beer fermentation, the enzyme is ultimately responsible for converting pyruvate to lactic acid, which provides a sour flavor to the final product. The modified yeast allows for a more predictable fermentation compared to other methods of producing sour beer. Other methods to produce sour beer may use bacteria and/or wild yeasts that are difficult to control. The percentage of acids in a beer, primarily lactic and acetic acid, determines the sourness and there are a number of sour beers produced that have similar amounts of lactic acid compared to this strain (see Figure 1).

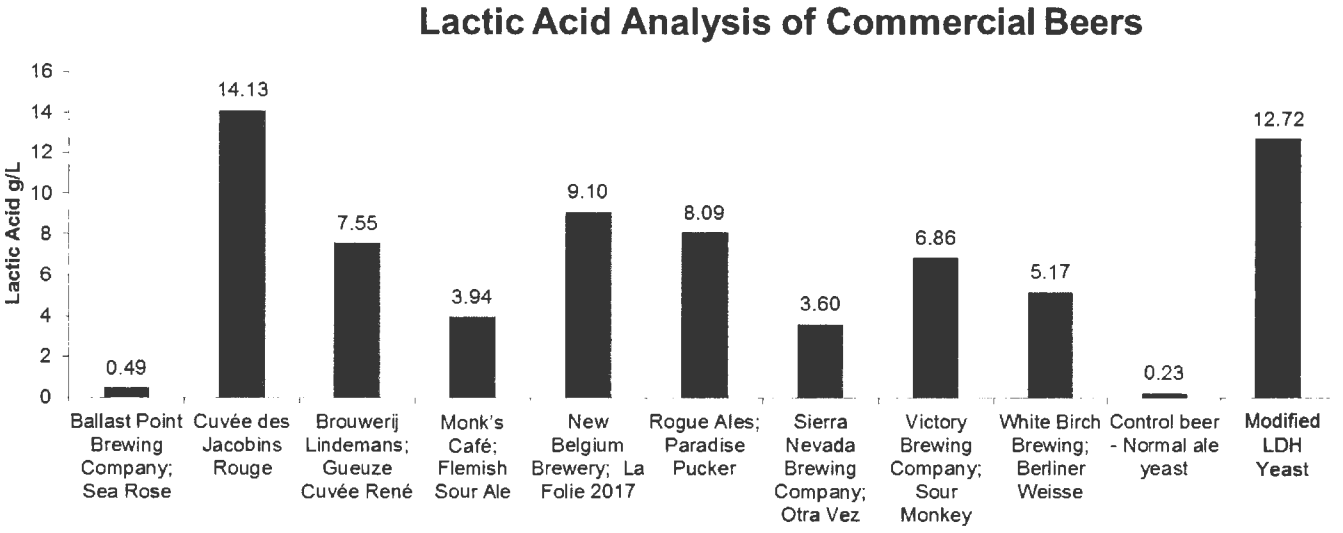

Figure 1. Comparison of lactic acid levels (g/L) in various beer strains available commercially. The modified LDH yeast is the subject of this GRAS conclusion.

2.3.2 Host Microorganism

The *Saccharomyces cerevisiae* parent yeast was isolated from a commercial sample of brewing yeast used for beer production. The parent strain has been used in beer production for over 20 years.

2.3.3 Construction

The strain was constructed using one genetic modification. The molecular tools and practices used during the construction of the production strain are standard to the field of biotechnology and yeast genetics. The genetic modification techniques utilized to develop this modified strain relies upon directed integration to insert the gene at a specific and known site within the yeast chromosome. The direct integration approach creates strains with integration events that are stable and easy to characterize. Chromosomal integration, by its very nature, reduces the probability of any mobilization of the heterologous DNA and enhances strain stability relative to other approaches.

The expression cassette for the lactate dehydrogenase was directly integrated into the chromosome of the host strain by recombination. Chromosomal integration, by its very nature, reduces the probability of any mobilization of the heterologous DNA and enhances strain stability relative to other approaches. The PCR products used to transform the host included the *Rhizopus oryzae* LDH gene encoding lactate dehydrogenase, under the regulation of the native *S. cerevisiae* ADH1 promoter and PDC1 terminator. The genetic construction was evaluated for the incorporation of the desired functional genetic information. Based on whole genome next-generation sequencing, PCR analyses, and phenotypic characterizations, we confirmed that the LDH gene in the genetic construction had integrated at the targeted locus in the final production strain. No genes encoding for virulence factors, protein toxins or enzymes involved in the synthesis of mycotoxins or any other toxic or undesirable substances are expected based on our knowledge of the strain, the lactate dehydrogenase sequence, and the promoters and terminators.

2.3.4 Stability & Genetic Transfer Capability of Introduced DNA Sequences

The inserted DNA is integrated into the *Saccharomyces cerevisiae* chromosome resulting in transformants that are mitotically stable. Genetic transfer of the inserted DNA to other organisms is poor because the chromosomal integration severely limits the mobility of the inserted DNA.

Mascoma confirmed the strain stability over 100 generations by serially passaging the strain in YPD medium. PCR was performed to confirm stability of the engineered site. Similarly, the parental strain was also passaged as a control and all of the genotyping by PCR analyses were as expected, indicating no loss in the genetic stability. The phenotype of the 100 passage strain was analyzed. The strain produced equivalent lactic acid in an overnight YPD media to the initial non-passaged strain. Therefore, the genotype and phenotype of the production strain is stable.

2.3.5 Antibiotic Resistance Genes

During construction of the engineered strain, only a single plasmid was used during the transformation step, which contained the hygromycin resistance gene. However, this plasmid was only used as a co-transformation aid and no genetic material was integrated into the cell and the plasmid was quickly cured during the subsequent plating and passaging of the transformant. Confirmation of the plasmid removal and integration of the expression cassette were confirmed by PCR genotyping and dilution plating onto selective medium. Therefore, confirmation of removal of any antibiotic resistance genes was confirmed and no antibiotic resistance was conferred to the modified strain.

2.3.6 Absence of the Production Organism in the Final Product

Typically, in the commercial production of alcoholic beers, yeast is removed after fermentation either by settling and/or filtering and the product is pasteurized, which inactivates and removes the yeast in the final product. However, in some craft breweries, a secondary fermentation is performed on the beer in casks or bottles after the initial fermentation by adding additional sugar. In some cases, the brewer will remove the original yeast used in fermentation and add another type of yeast specifically for this purpose. In a small number of cases, however, the brewer may choose to reuse a portion of the yeast from the primary fermentation

(https://beerandbrewing.com/dictionary/iKSxvCoDdk/bottle-conditioning/). In these applications, the modified *Saccharomyces cerevisiae* will be replacing yeast from other available commercial sources in the fermentation of beer.

2.4 Manufacture of the Production Organism

2.4.1 Manufacturing of the Yeast

The manufacturing process for the production of modified *Saccharomyces cerevisiae* strain containing lactase dehydrogenase starts with a traditional baker's yeast process (Reed, 1982; Chen & Chiger, 1985; Rose & Vijayalakshmi, 1993; Plomp, 1999). The genetically modified yeast product is produced in accordance with with current good manufacturing practices for food (cGMP). A HACCP (Hazard Analysis Critical Control Points) plan, which includes ensuring microbiological purity, is employed during the entire production process. The production is conducted at a fermentation facility with established procedures and equipment suitable for large-scale contained production of bioengineered *Saccharomyces cerevisiae*. Physical inspection and the appropriate microbiological and fermentation analyses are conducted to confirm strain identity and functionality in application, ensuring that the yeast product meets the finished product specifications. These methods are based on generally available and accepted methods used for the production of microbial production organisms and the production of microbial enzymes (Stanbury and Whitaker, 1984).

2.4.2 Raw Materials

The raw materials used in the fermentation and recovery processes for the yeast product are standard food grade ingredients used in traditional baker's yeast production. The raw materials include a source of carbon, which are typically molasses and other nutrients (essential elements and vitamins). All raw materials conform to Food Chemicals Codex (FCC) specifications except for those raw materials that do not appear in the FCC. For those that do not appear in FCC specifications, suitable ingredients are used and internal specifications are established to meet the ones set forth by the FCC requirements. Certain minor components such as trace minerals and vitamins used in the fermentation media and acids used for pH adjustment are food grade.

2.4.3 Lab Stage

Yeast propagation is initiated from frozen cultures maintained at -80°C in glycerol. A working stock culture derived from the master cell bank is used to start the propagation. The frozen working stock culture is first inoculated under strict sterile conditions into a flask of 5 – 10 L of sterile medium (autoclaved). This flask is cultivated in the laboratory to increase the numbers of growing cells prior to inoculating the culture into the production vessels.

2.4.4 Fermentation

The yeast from the flask is inoculated into a larger propagation tank, typically 2,100 L working volume. The culture is sequentially transferred into increasing fermentor volumes, depending on

the desired amount of product. The final fermentation is fed with carefully controlled amounts of sugar and air to achieve the maximum output of yeast product.

To prevent contamination of foreign microorganisms, all equipment is carefully operated, cleaned, and maintained including steam sterilization of primary ingredients. The fermentation vessels are cleaned in place (CIP) with acid and base, then rinsed with water until a neutral pH is reached before and between production batches. Throughout the fermentation steps, key control parameters are monitored to confirm proper growth and ensure consistent production. Temperature, pH, and aeration rate are monitored and controlled. The fermentor off-gas is monitored for ethanol production, and the feeding rate of molasses is adjusted to provide the optimal growth with minimal ethanol production.

2.4.5 Recovery and Formulation of the Finished Product

The recovery process is initiated upon completion of fermentation. Yeast cells are centrifuged from the fermentation broth. The yeast is then washed to remove remaining non-yeast soluble solids, leading to a liquid yeast slurry with 20- 30% solids (200-300 g dry weight/kg). The yeast slurry can be sold as liquid yeast and is stable up to 6 months.

If a dry product is desired, the yeast is filtered and fed to a rotary vacuum filter which is coated with large starch granules. The yeast is drawn onto the granules, while water moves through the rotating drum and a cold water spray washes the yeast. A blade removes the yeast in a thin layer from the starch granule filter bed. The yeast from the rotary vacuum filter is then extruded and mixed with an emulsifier, such as 0.2% sorbitan monostearate. Instant Dry Yeast (IDY) is produced by extruding the yeast in continuous 'spaghetti-like' strands through holes in a perforated plate and then fed to a dryer to further reduce its moisture content to approximately 5% w/w. Fluidized bed or air lift dryers are used to produce IDY in the form of small strands. The dry product will be stable up to 2 years.

2.5 Product Specifications

The specifications for liquid yeast and dry yeast are measured on every batch prior to Quality Control (QC) release (See Table 2 and 3). The total bacteria and wild yeast protocols are based on American Society of Brewing Chemists (ASBC) methods of analysis. The liquid and dry yeast do not contain any major food allergens from the fermentation media.

Table 2. Liquid yeast product specifications

Parameter	Specification Range
Solids (Dry Matter)	20 - 30%
Total Viable Cells	> 95%
Viable cell count	> 100 x 10^6 cells/ml
Total Bacteria	< 1 per 10^6 yeast cells
Wild Yeast	< 1 per 10^6 yeast cells

Table 3. Dry yeast product specifications

Parameter	Specification Range
Dry Weight	93 - 96%
Viability	> 5 x 10^9 cells per gram
Total Bacteria	< 1 per 10^6 yeast cells
Wild Yeast	< 1 per 10^6 yeast cells
Coliforms	< 100 per gram
Escherichia coli	< 10 per gram

If a fermentation batch is determined to be not in compliance with QC specifications, it will be rejected. In addition, a fermentation assay is performed on the product to confirm the phenotype of the strain. **Table 4** contains data for three batches of the liquid yeast and **Table 5** contains data for three batches of the dry yeast based on the specifications above.

Table 4: Analytical data for three batches of liquid yeast with lactate dehydrogenase

Parameter	Specification Range	Manufacturing Lot		
Solids (Dry Matter)	20 - 30%	21.00%	27.50%	28.00%
Total Viable Cells	> 95%	99.80%	96.85%	95.70%
Viable cell count	>100 x 10^6 cells/ml	4.32 x 10^9 cells/ml	6.7 x 10^9 cells/ml	4.95 x 10^9 cells/ml
Total Bacteria	< 1 per 10^6 yeast cells	< 1 per 2.2 x 10^8 yeast cells	<1 per 1.3 x 10^8 yeast cells	<1 per 9.9 x 10^7 yeast cells
Wild Yeast	< 1 per 10^6 yeast cells	< 1 per 2.2 x 10^8 yeast cells	<1 per 3.4 x 10^8 yeast cells	<1 per 2.5 x 10^8 yeast cells

Table 5: Analytical data for three batches of dry yeast with lactate dehydrogenase

Parameter	Specification Range	Manufacturing Lot		
Dry Weight	93 - 96%	95%	94%	93%
Viability	> 5 x 10^9 cells per gram	5.6 x 10^9	1.34 x 10^{10}	7.8 x 10^9
Total Bacteria	< 1 per 10^6 yeast cells	<1 x 10^6 yeast cells	<1 x 10^6 yeast cells	<1 x 10^6 yeast cells
Wild Yeast	< 1 per 10^6 yeast cells	< 1 per 10^6 yeast cells	< 1 per 10^6 yeast cells	< 1 per 10^6 yeast cells
Coliforms	< 100 per gram	< 10 per gram	<10 per gram	<10 per gram
Escherichia coli	< 10 per gram	< 10 per gram	<10 per gram	<10 per gram

2.6 Application and Use Levels

2.6.1 Mode of Action

LDH can make pyruvate from lactate or lactate from pyruvate, as it converts NAD+ to NADH and back. The *S. cerevisiae* strain has been genetically modified to express a lactate dehydrogenase enzyme which catalyzes the conversion of pyruvate to lactate or the reverse action of lactate to pyruvate. Fermentation processes that produce fermented foods for human consumption are now well established, but this is only in modern times. LDH and lactic acid were unknown for thousands of years without an appreciation or an understanding of these underlying mechanisms (Chilton *et al.*, 2015; Franz *et al.*, 2014; Lücke, 1994). A 1992 overview of traditional fermented foods around the world including kimchi, tempe, yogurt, miso, sauerkraut and many others fails to mention LDH (Steinkraus, 1992). Lactic acid fermentation also produces sour beer. Lactic acid is a component in the production of these beers, including lambics and Berliner Weisses. Berliner Weisse is a cloudy, sour beer that dates back to at least the 16th century. The fermentation process includes a mixture of yeast and lactic acid bacteria, which gives the beer its distinctive taste. Lambic beer is brewed in Brussels and is fermented spontaneously by being exposed to native wild yeasts and bacteria (https://beerandbrewing.com/the-sour-beer-spectrum/).

The literature reports on the potential of genetic technology to improve brewing, wine making and baking yeasts (Dequin, 2001). Sauer *et al.* overview yeast research including the heterologous LDH activity that directs pyruvate to lactic acid (2010; see Figure 1, page 232). In beer fermentation, the enzyme is ultimately responsible for converting pyruvate to lactic acid, which provides a sour flavor to the final product.

LDH is a key enzyme in anaerobic respiration and in the production of lactic acid. Anaerobic respiration is the conversion of pyruvate into lactic acid in the absence oxygen. This pathway is important to glycolysis in two main ways. The first is that if pyruvate were to build up, glycolysis and thus the generation of ATP would slow. The second is anaerobic respiration allows for the regeneration of NAD+ from NADH. NAD+ is required when glyceraldehyde-3-phosphate dehydrogenase oxidizes glyceraldehyde-3-phosphate in glycolysis, which generates NADH. Lactate dehydrogenase is responsible for the anaerobic conversion of NADH to NAD+.

$$\text{Pyruvate}^- + \text{NADH} + \text{H}^+ \leftrightarrow \text{Lactate}^- + \text{NAD}^+$$

Enzymes found in nature have been used since ancient time in the production of food and beverage products, such as cheese, sourdough, beer, wine and vinegar. Records of the alcoholic fermentation of barley to produce beer and grapes to make wine are over 5000 years old (Campbell-Platt, 1994). Dequin *et al.* reports on the acidification of grape musts by *S. cerevisiae* wine yeast strains that are genetically engineered to produce lactic acid (1999). Fermentation of lactic acid has wide applications in the food and beverage industries worldwide. It is estimated that fermented foods and beverages worldwide provide typically about one-third of all food intake (Campbell-Platt, 1994).

Production of lactic acid is commonly carried out by the lactic acid bacteria, *Lactobacillus spps.*, for production of cheese, yogurt, sauerkraut, bread, and kefir, and for imparting a peculiar sour

taste to such food items. All beverage industries use the above described fermentation mechanism to produce wines, alcohol, beer, brandy, and other beverages. Lactic acid products are high in vitamins and essential nutrients.

Stewart reviews beer production using *Saccharomyces* species and discusses the fermentation process including flocculation and the importance of wort composition (2016). To produce alcoholic beer, grains, typically barley, are processed by milling, mashing, lautering, boiling and finally separating in a wort separating stage. After the boiled wort is cooled, the modified yeast is added in a fermentation vessel to convert the liquid sugary wort into carbon dioxide and alcohol, along with various yeast metabolites that contribute flavor to the finished beer. The modified yeast encoded with the lactate dehydrogenase enzyme will produce alcohol and impart a sour flavor to the beer during the fermentation and allow for a more predictable fermentation compared to other methods of producing sour beer. Other methods to produce sour beer may use bacteria and/or wild yeasts that are difficult to control. After fermentation, the beer will be matured for a few weeks or months to allow further development of flavors and a smooth finish. Typically in the commercial production of alcoholic beers, the product is pasteurized and filtered, which inactivates and removes the yeast in the final product. However, in some craft breweries, the yeast is not completely removed from the initial fermentation. The remaining yeast may be used in a secondary fermentation, although normally a yeast specific for re-fermentation is used, so it is significantly less likely that the modified yeast will remain in the product.

2.6.2 Use and Use Levels

The modified yeast is used as a processing aid in the fermentation of beer. The yeast with the lactate hydrogenase enzyme is used in brewing at levels to achieve the desired effects for alcohol and lactic acid content (sourness) according to good manufacturing practices. The recommended pitching rate of the yeast is 50 – 100 g/hL to achieve a minimum of 2.5 to 5 million cells/mL at the beginning of the beer fermentation.

2.6.3 Enzyme Residues in the Final Food

The potential exposure of humans to the modified *Saccharomyces cerevisiae* strain and the expressed enzyme is limited by the beer production process itself, whereby the processing of the beer either removes the yeast or makes the yeast non-viable. Normally in commercial beer manufacturing, the alcohol product is pasteurized and filtered at the end of processing (https://beerandbrewing.com/dictionary/5MrUJTLOWe/filtration/; https://beerandbrewing.com/dictionary/edvVKFchSZ/pasteurization/). The theoretical maximum exposure is therefore limited by the fraction of the yeast in the beer, which is typically made non-viable or removed by pasteurization and/or filtration. There may be some craft brewers who will not remove all of the yeast from the process and may perform a secondary fermentation with the yeast. However, yeast and enzymatic activity will be halted by the depletion of the substrate, such as glucose, during the process whether or not the yeast is pasteurized or removed from the product. Pritchard (1973) studied the LDH enzyme from *Rhizopus oryzae* and concluded that under conditions of low glucose, LDH activity is negligible. The yeast and enzyme do not exert a function in the final product.

Craft brewers may opt to not filter beer. Instead of filtering the beer, yeast cells are allowed to settle and the liquid is decanted from the pelleted yeast mass. This process removes the majority of cells as the beer is unpalatable otherwise. In these cases where craft brewers do not completely remove the yeast with the LDH enzyme from the process, digestion of the yeast will largely inactivate or denature the enzyme. Among the best-known proteolytic enzymes are those that reside in the digestive tract, including trypsin. In one study, Clausen and Ovlisen (1965) isolated LDH from human spermatozoa and semen, which was logarithmically inactivated by the enzyme trypsin. In addition, the pH of the gastric acid in the stomach is 1.5 to 3.5 and LDH enzymatic activity has been shown to decline with low pH. Vallee and Williams (1975) studied LDH from beef and determined that at a pH of 2.0 or 3.0, beef B4 lactate dehydrogenase rapidly loses enzymatic activity within 15 seconds of exposure. In addition, Prichard (1973) reported that the optimum pH for LDH activity from *Rhizopus oryzae* is 6.0 to 8.6. Thus, any LDH remaining will be inactivated and denatured during digestion.

In section 6.2, the safety of *Saccharomyces cerevisiae* is discussed. It is noteworthy that this yeast has a long history of safe use and a collection of safety studies conducted over the years provide additional support.

3. Dietary Exposure

Saccharomyces cerevisiae is well-established as a safe food ingredient. Its use spans thousands of years. The yeast and its properties are so well documented and understood that it makes *Saccharomyces cerevisiae* an ideal host for safely expressing enzymes. In addition, the expressed enzyme lactate dehydrogenase is widely distributed in nature and is found in nearly all living cells, including vertebrates, plants and bacteria. LDH has been widely studied in humans and is found in healthy human cells, including the heart, kidney, liver, muscles and blood. It has been used clinically as a biomarker for diseases and other pathological conditions in particular tissues.

3.1 Estimates of Human Consumption

As discussed above, the modified *Saccharomyces cerevisiae* producing the lactate dehydrogenase enzyme from *Rhizopus oryzae* is comparable to non-modified yeast. The yeast will be used as a replacement for other commercial yeasts in the beer manufacturing process. Humans consuming the enzyme and the inactivated modified yeast are not expected to present any safety risk.

The modified *Saccharomyces cerevisiae* yeast is intended for use as yeast in brewing and does not impart any nutritional or safety effects in beer consumed by humans.

In addition to the common use of *S. cerevisiae* in human food as evidenced by references to yeast in sections 172.896, 172.898, 172.325, 172.590 and 184.1983 of the Code of Federal Regulations, Vol 21. FDA has had no questions on GRAS Notifications for a number of bioengineered *Saccharomyces cerevisiae* for the direct addition to human food. See Section 6.2.

Still, exposure estimates for modified *Saccharomyces cerevisiae* are provided in the event that a craft brewer decides not to pasteurize or remove all of the yeast. The intake will be based on the

amount of the modified yeast after fermentation before final yeast removal as a "worst case" scenario.

The following maximum potential exposure estimates based on average potential beer intake calculations for modified *Saccharomyces cerevisiae* yeast. It is assumed that only men and women over the age of 21 will be exposed to the yeast due to the legal drinking age in the United States.

Assumptions for Calculations:

- Craft brewery: Maximum of 1 x 10^6 yeast cells/ml in beer product.[1]
- Consumption (Guenther *et al.*, 2010, based on NHHANES studies, 2003-2006) of beer for people over the age of 21; Weight (McDowell *et al.*, 2008, National Health Statistics Report):

Population	Daily beer consumption (g)	Daily beer consumption	Weight (kg)
Men	425	417	88.3
Women	137	134	74.7

- Maximum protein in a yeast cell is 6.0 x 10^{-12} g/cell (Siwiak & Zielenkiewicz, 2010).
- Assume 5% of cell protein is LDH.[2]
- Maximum activity of LDH is 9.2 units/mg protein or 9200 units/g (Skory, 2003; Wu *et al.*, 2011).[3]

LDH Exposure:

- 9200 units/g LDH protein x [(6.0 x 10^{-12})g yeast protein / yeast cell] x [0.05 g LDH protein/g yeast protein] = (2.8 x 10^{-9})units LDH / yeast cell

[1] Based on the final yeast in beer at the end of fermentation before final yeast removal, which can range from 1 – 3 x 10^6 per ml (Boulton & Quain, 2001, p.244).

[2] High expression of yeast proteins, from 4 – 15% of the total cell protein, have been examined (Alberghina *et al.* 1991; Martinez *et al.*, 2015). This strain has not been optimized for LDH expression, therefore it's reasonable to expect the total amount of LDH protein in the cell is ≤5% of the total yeast protein.

[3] Skory (2003) and Wu *et al.* (2011) attempted to maximize lactic acid production. The maximum activity of LDH is based on the maximum amount of protein measured.

Human Consumption:

A. Men > 21 years of age:

- 418 ml beer/day x [(1 x 10^6)yeast cells/ml] / [88 kg bw] =

  4.7 x 10^6 cells/ kg bw/ day

- 4.7 x 10^6 cells/ kg bw/ day x (2.8 x 10^{-9})units LDH / yeast cell =

  0.013 units LDH/ kg bw/day

B. Women > 21 years of age:

- Using the same information for women, exposure is **0.005 units LDH/ kg bw/day**

The amount of maximum potential exposure to LDH in the "worst case" scenario, is extremely small. As mentioned in Section 2.1, the normal human range for LDH is 105 – 333 IU/l. In addition, the commercial brewer normally filters and/or pasteurizes the final beer product, removing the yeast and enzyme, which reduces or eliminates the human exposure to the yeast and enzyme.

3.2 Dietary Exposure to Any Other Substance Formed in or on Food

The lactate dehydrogenase enzyme in the modified *S. cerevisiae* produces lactic acid during fermentation as flavoring in beer production. Lactic acid is considered GRAS as a direct food ingredient when used in food as a flavoring agent with no limitation other than current good manufacturing practice. (21 CFR § 184.1061)

3.3 Dietary Exposure to Contaminants or Byproducts

Monitoring of fermentation parameters may include pH, aeration, temperature, and off-gas production. The measured values of these parameters are constantly monitored during the fermentation process. The values indicate whether sufficient biomass has been developed and the fermentation process evolves according to plan. Deviations from the pre-defined values lead to adjustment, ensuring an optimal and consistent process.

4. Self-Limiting Levels of Use

There are no proposed restrictions on the use of the modified *Saccharomyces cerevisiae* especially because the yeast should be used in brewing consistent with good manufacturing practices. See Section 2.5. In addition, the self-limited levels of use are primarily flavor as customers are unlikely to use more yeast than is needed to achieve the technical effects due to an increasingly unacceptable organoleptic profile.

Based on the full safety assessment, Mascoma concludes that there is a reasonable certainty of no harm to humans consuming beer containing the bioengineered *Saccharomyces cerevisiae*.

5. Experience Based on Common Use in Food before 1958 – NOT APPLICABLE

6. Narrative

This safety assessment of our genetically modified yeast strain to be used in beer production includes an evaluation of the safety of the production organism, the host organism, the enzyme, the donor, the manufacturing process and consideration of the dietary exposure. Each of these topics is addressed below.

6.1 Safety of the Production Organism *Saccharomyces cerevisiae*

Safety of the production organism is the prime consideration in assessing the probable degree of safety of the resulting processing aid used in food (Pariza & Foster, 1983; Pariza & Johnson, 2001). The host strain used for modified *Saccharomyces cerevisiae* strain producing the lactate dehydrogenase is a non-modified brewer's yeast. This *Saccharomyces cerevisiae* strain was selected because of its use as a commercial strain in brewer's yeast production and it's similarity to other brewing yeast strains.

Saccharomyces cerevisiae has an extensive history of safe use for over thousands of years in connection with food and feed, primarily the fermentation and preservation of foods. *Saccharomyces cerevisiae* yeast has been used by the ancient Egyptians, Romans, Hebrews and Greeks in fermentation processes for the production of wine, bread, and beer. Commercialized yeast cell preparations and associated nutrients such as proteins, amino acids, vitamins, minerals and trace elements are used as food supplements or in the production of medical products (Moyad, 2007; Moyad, 2008).

Saccharomyces cerevisiae is ubiquitous, is commonly found in our daily lives as it is in the air we breathe, and grows naturally on foods, such as fruits and vegetables especially ones with high fermentable sugars that we consume daily. *Saccharomyces cerevisiae* is a common colonizer of mucosal surfaces and part of the normal flora of the gastrointestinal tract, the respiratory tract, and the vagina (Salonen et al., 2000; Munoz et al., 2005). A summary of the extensive benefits of *S. cerevisiae* on human health has been reviewed (Moslehi-Jenabian et al., 2010). Fleet notes that humans consume large quantities of yeasts without adverse impact on human health, which is unlike bacteria and viruses (2007). Recent studies, such as the acute and subacute toxicity testing of yeast hydrolysate from *Saccharomyces cerevisiae*, show very low toxicity providing additional support of the safety of the yeast as a probiotic (Jung *et al.*, 2010). This further supports the conclusion that *Saccharomyces cerevisiae* yeast is non-pathogenic and non-toxigenic.

Over 2.5 million tons of yeasts are commercially produced each year worldwide making *Saccharomyces cerevisiae* the most widely used microorganism (Halász & Lásztity, 1991; Boekhout & Robert, 2003; Fleet, 2006). About 150 different wine yeast strains, mainly *S. cerevisiae*, are commercially available (Branduardi *et al.*, 2008). The genome of *Saccharomyces cerevisiae* has been completely sequenced disclosing about 6,000 genes that are identical or similar to human genes (Goffeau *et al.*, 1996; Branduardi *et al.*, 2008). *Saccharomyces cerevisiae* is the microorganism of choice for research and industrial use as it is easy to

manipulate and grow with the capability of producing high, predictable yield that can be well controlled and scaled for industrial use (Ostergaard *et al.*, 2000).

6.2 Regulatory Overview

Extensive regulatory approvals support the safety of *Saccharomyces cerevisiae* for diverse uses including food, feed, and pharmaceutical applications.

6.2.1 US Regulatory Overview

6.2.1.1 Code of Federal Regulations (CFR)

Listings of *Saccharomyces cerevisiae* in the Code of Federal Regulations (C.F.R.) are extensive and include:

- Baker's yeast extract (21 C.F.R. § 184.1983)
- Baker's yeast protein (21 C.F.R. § 172.325);
- Yeast-malt sprout extract (21 C.F.R. § 172.590);
- Dried yeast as an ingredient in food (21 C.F.R. § 172.896);
- Baker's yeast glycan (21 C.F.R. § 172.898);
- Direct addition of food grade baker's yeast (*S. cerevisiae*) in
    - Eggs (dried eggs – 21 C.F.R. § 160.105
    - Dried egg whites – 21 C.F.R. § 160.145
    - Dried egg yolks – 21 C.F.R. § 160.185
- Since 1902, autolyzed yeast and cell membranes of yeast have been used for treatment of wine (27 C.F.R. § 24.246).

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

6.2.1.2 GRAS

In addition to the common use of *Saccharomyces cerevisiae* in human food, FDA has had no questions on GRAS Notifications for a number of modified *Saccharomyces cerevisiae* for the direct addition to human food. These include:

- GRN 744 Steviol Glycosides with a High Rebaudioside M Content Produced by Microbial Fermentation

- GRN 626 Steviol glycosides produced in *Saccharomyces cerevisiae* strain S288C as a general use sweetener in foods and beverages

- GRN 422 *Saccharomyces cerevisiae* transformed with three copies of the *S. cerevisiae* ASP3 gene encoding for asparaginase
- GRN 350 *Saccharomyces cerevisiae* strain P1Y0 for use as a starter culture for alcoholic beverage fermentation
- GRN 175 *Saccharomyces cerevisiae* strain ECMo01 with enhanced expression of urea amidolyase—for use in fermented beverages
- GRN 120 *Saccharomyces cerevisiae* strain ML01 carrying a gene encoding the malolactic enzyme from *Oenococcus oeni* and a gene encoding malate permease from *Schizosaccharomycespombe*—for use in winemaking as a yeast starter culture for grape must fermentation
- GRN 88 Invertase enzyme preparation from *Saccharomyces cerevisiae* and lactase enzyme preparation from *Kluyveromyces marxianus*—for use in foods in general as an enzyme

6.2.1.3 National Institutes of Health (NIH)

The NIH Guidelines for Research Involving Recombinant DNA Molecules considers *Saccharomyces cerevisiae* a safe host organism and qualifies as a Risk Group 1 agent as it is not associated with disease in healthy adult humans under its Basis for the Classification of Biohazardous Agents by Risk Group (U.S. DHHS, 2016– Appendix C-III).

As EPA recognized in its Final Risk Assessment of *Saccharomyces cerevisiae* (February 1997; U.S. EPA, 1997 - p. 9), "[m]any scientists believe that under appropriate conditions any microorganism could serve as an opportunistic pathogen." The Agency concluded that *S. cerevisiae* has an extensive history in food processing and neither it nor other closely related species "has been associated with pathogenicity toward humans or has been shown to have adverse effects on the environment" (p.2). Specifically, with respect to human exposure, EPA concluded on p. 3 of the Final Risk Assessment that:

> "There are individuals who may ingest large quantities of *S. cerevisiae* every day, for example, people who take the yeast as part of a "health food" regimen. Therefore, studies were conducted to ascertain whether the ingestion of large numbers of these yeasts might result in either colonization, or colonization and secondary spread to other organs of the body. It was found that the installation of very large numbers of *S. cerevisiae* into the colons of animals would result in both colonization and passage of the yeasts to draining lymph nodes. It required up to 10^{10} *S. cerevisiae* in a single oral treatment to rats to achieve a detectable passage from the intestine to the lymph nodes (Wolochow *et al.*, 1961). The concentrations of *S. cerevisiae* required were well beyond those that would be encountered through normal human daily exposure."

EPA concluded that: "*Saccharomyces*, as a genus, present low risk to human health or the environment. Criteria used to differentiate between species are based on their ability to utilize specific carbohydrates without relevance to pathogenicity. Nonetheless, this risk assessment applies to those organisms that fall under the classical definition of *S. cerevisiae* as described by

van der Walt (1971)." The modified *S. cerevisiae* strain falls under the classical definition described by van der Walt (1971).

Thus, FDA, NIH, and EPA have concluded the safety of *Saccharomyces cerevisiae* as a non-pathogenic microorganism.

6.2.2 European Food Safety Agency (EFSA) Regulatory Overview

According to EFSA, yeasts used in food production, particularly bakers/brewer's yeast, are considered among the safest of microorganisms (EFSA, 2007, 2018). *Saccharomyces cerevisiae* is one of the safest microorganisms used in food and feed production and has been designated Qualified Presumption as Safe (QPS) status in Europe, which indicates that no additional safety assessment is needed according to established guidelines (EFSA, 2007, 2008). A recent safety review by EFSA continues to support the QPS status of *Saccharomyces cerevisiae* (EFSA, 2018). One example of a feed approval is the inactivated and dried selenized yeast produced by *Saccharomyces cerevisiae* providing selenium, an essential trace element, in an organic form as a nutritional additive for use in poultry, pigs, and bovines (EFSA, 2006.) EFSA reviewed the safety and efficacy of selenium-enriched yeast (*Saccharomyces cerevisiae* CNCM I-3399) for all animal species (EFSA, 2009).

EFSA notes that "[r]are opportunistic infections have been caused by *Saccharomyces cerevisiae*," and EFSA maintains its QPS (Qualified Presumption as Safe) status (EFSA, 2008). EFSA provides additional clarification stating, "*Saccharomyces cerevisiae*, subtype *boulardii* is contraindicated for patients of fragile health, as well as patients with a central venous catheter in place. A specific protocol concerning the use of probiotics should be formulated" (EFSA, 2008, Table 4, pp.21, 43). Even with the infrequent cases of fungemia associated with *S. boulardii*, McFarland discusses contraindications and precautions and recommends closely monitoring adult immuno-compromised patients and catheter use, especially with unexplained fever and notes that some recommend not giving *S. boulardii* to immuno-suppressed patients or those with central catheters to reduce the risk of fungemia (Buts, 2009; McFarland, 2010).

6.2.3 Food Standards Australia New Zealand

Saccharomyces cerevisiae is recognized as a safe microorganism for processing aids (Schedule 18)[4].

[4] Available at http://www.foodstandards.gov.au/code/Documents/Sched%2018%20Processing%20aids%20v159.pdf.

6.2.4 Health Canada

Saccharomyces spp. is listed as a source microorganism for the production of invertase and lactase[5].

6.2.5 Regulatory Overview of Pharmaceuticals

As of January 2009, twenty-eight of the 151 protein-based recombinant pharmaceuticals that have been approved by the FDA and EMEA (European Medicines Agency) were produced in *Saccharomyces cerevisiae* (Ferrer-Miralles *et al.*, 2009; Huang *et al.*, 2010). The first vaccine effective against hepatitis B was produced intracellularly in recombinant *S. cerevisiae* (McAleer *et al.*, 1984; Çelik & Çalık, 2012). Insulin, hepatitis B surface antigen, GM-CFS, hirudin, platelet-derived growth factor are among other pharmaceuticals produced by *S. cerevisiae* (Demain & Vaishnav, 2009).

6.2.6 Safety Studies

Pineton de Chambrun, *et al.* (2015) conducted a randomized clinical trial of *Saccharomyces cerevisiae* versus a placebo in the irritable bowel syndrome. 179 adults with irritable bowel syndrome were randomized to receive once daily 500 mg of *Saccharomyces cerevisiae*, delivered by one capsule (n = 86, F: 84%, age: 42.5 ± 12.5), or placebo (n = 93, F: 88%, age: 45.4 ± 14) for 8 weeks followed by a 3-week washout period. After a 2-week run-in period, cardinal symptoms (abdominal pain/discomfort, bloating/distension, bowel movement difficulty) and changes in stool frequency and consistency were recorded daily and assessed each week. A safety assessment was carried out throughout the study. The proportion of responders, defined by an improvement of abdominal pain/discomfort, was significantly higher ($p = 0.04$) in the treated group than the placebo group (63% vs 47%, OR = 1.88, 95%, CI: 0.99-3.57) in the last 4 weeks of treatment. A non-significant trend of improvement was observed with *Saccharomyces cerevisiae* for the other symptoms. *Saccharomyces cerevisiae* was well tolerated and did not affect stool frequency and consistency.

Schauss, *et al.* (2012) reported on a safety evaluation of a food-grade, dried fermentate (EpiCor) of *Saccharomyces cerevisiae*. Studies included the following assays: bacterial reverse mutation, mouse lymphoma cell mutagenicity, mitogenicity assay in human peripheral lymphocytes, and a cytochrome P450 ([CYP] CYP1A2 and CYP3A4) induction assessment as well as 14-day acute, 90-day subchronic, and 1-year chronic oral toxicity studies in rats. No evidence of genotoxicity or mitogenicity was seen in any of the in vitro or in vivo studies. The CYP assessment showed no interactions or inductions. No toxic clinical symptoms or histopathological lesions were observed in the acute, subchronic, or chronic oral toxicity studies in the rat. Results of the studies performed indicate that EpiCor does not possess genotoxic activity and has a low order of

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[5] Invertase, http://webprod.hc-sc.gc.ca/nhpid-bdipsn/ingredReq.do?id=4588&lang=eng; Lactase, http://webprod.hc-sc.gc.ca/nhpid-bdipsn/ingredReq.do?id=7307&lang=eng last accessed October 9, 2018.

toxicity that is well tolerated when administered orally. The no observable adverse effect level (NOAEL) was 1500 mg/kg body weight (bw)/d for the 90-day study and 800 mg/kg bw/d for the 1-year study, for the highest doses tested.

Pereyra *et al.* (2014) reports on the probiotic *Saccharomyces cerevisiae* RC016 and test its ability to reduce genotoxicity caused by dietary aflatoxins (AFs). The probiotic was orally administered to Wistar rats. Six groups (n = 6) were arranged: feed and probiotic controls, two levels of AFs-contaminated feed and two treatments including both the probiotic and the toxin. Genotoxicity and cytotoxicity were evaluated with the bone marrow micronuclei assay and the comet assay and internal organs were macroscopically and microscopically examined. The tested *S. cerevisiae* strain did not cause genotoxicity or cytotoxicity in vivo, and it was able to attenuate AFs-caused genotoxicity. *Saccharomyces cerevisiae* RC016 did not cause any impairment on the rats' health and it showed no negative impact on the weight gain. Moreover, RC016 improved zootechnical parameters in AFs-treated animals. The beneficial effects were likely to be caused by adsorption of AFs to the yeast cell wall in the intestine and the consequent reduction in the toxin's bioavailability. It was concluded that dietary administration of RC016 does not induce genotoxicity or cytotoxicity to rats.

Jung *et al.* (2010) showed that yeast hydrolysate from *Saccharomyces cerevisiae* had very low toxicity in rat studies. This study was designed to test yeast hydrolysate in 10-30 kDa molecular weight for use as a dietary supplement by assessing its acute and subacute oral toxicity in female and male Sprague-Dawley (SD) rats. The single oral dose of the hydrolysate at 5000 mg/kg did not produce mortality or significant changes in the general behavior and gross appearance of the internal organs of rats. In subacute toxicity study, the hydrolysate was administered orally at a dose of 1000 mg/kg/day for a period of 14 days. The satellite group was treated with the hydrolysate at the same dose and the same period and kept for another 14 days after treatment. There were no significant differences in organ weights between control and treated group of both sexes. Hematological analysis and blood chemistry revealed no toxicity effects of *Saccharomyces cerevisiae* hydrolysate. Pathologically, neither gross abnormalities nor histopathological changes were observed. It was concluded that results showed that the hydrolysate has very low toxicity in the SD rat model.

Ardiani *et al.* reviews preclinical and clinical studies supporting the use of heat-killed whole recombinant *Saccharomyces cerevisiae* cells as therapeutic vaccines to treat cancer and infectious diseases (2010). Wansley *et al.* further notes that 'one of the reasons for interest in recombinant *Saccharomyces cerevisiae* as a vaccine vehicle is its lack of toxicity. Besides being inherently nonpathogenic, this particular species of yeast can be heat-killed before administration and has been shown to be safe in humans in several clinical trials, with maximum tolerated dose not reached (2008; Franzusoff *et al.*, 2005).

6.2.7 Conclusions

As summarized above, modern biotechnology has been delivering a wide range of safe products derived from *Saccharomyces cerevisiae* including food, beverages, feed, pharmaceuticals, enzymes, lipids and vitamins (Stewart & Russell, 1985; Bigelis, 1985; Gerngross, 2004; Redwan, 2007).

Based on the safety assessment, the modified *Saccharomyces cerevisiae* strain is a safe product based on the following key elements:

1) As described in Section 6.1, the host yeast *Saccharomyces cerevisiae* is a common human food and animal feed ingredient with a long history of safe use. *Saccharomyces cerevisiae* is used in a number of applications in human food, as a source of single cell protein, and as a processing aid in the production of alcohol.

2) As described in Section 6.2, bioengineered *Saccharomyces cerevisiae* strains have a long history of safe use in the production of food and feed enzymes and human pharmaceuticals. There have been no reports of isolates of *Saccharomyces cerevisiae* that produce toxins either against humans or animals.

3) As described in Section 6.4, the modified yeast strain meets the criteria of a safe production strain using the internationally accepted decision tree analysis originally proposed by Pariza and Johnson (2001).

4) As described in Section 3.1, the potential exposure of humans to the modified *Saccharomyces cerevisiae* strain, and the expressed enzyme is limited by the beer production process itself, whereby the processing of the beer either removes or makes the yeast non-viable. The theoretical maximum exposure is limited by the fraction of the yeast in the beer, which is typically killed or removed by pasteurization and/or filtration.

5) As described in Section 6.5.1, reports of *Saccharomyces cerevisiae* which may appear inconsistent with a GRAS determination, such as reports of this yeast having properties of an opportunistic pathogen, have been limited to immunocompromised individuals and is not expected to affect the safety profile of the strain.

6) As described in Section 6.5.2, reports of *Rhizopus oryaze* which may appear inconsistent with a GRAS determination, such as reports of this donor having properties of an opportunistic pathogen, have been limited to immunocompromised individuals and is not expected to affect the safety profile of the strain. In fact, based on the regulatory and safety reviews, *Rhizopus orzyae* is recognized as an acceptable donor and organism for the production of a variety of enzymes for use in food production. In addition, *Rhizopus oryzae* strains are often used in Asia for food fermentation to manufacture alcoholic beverages, ragi, or tempeh, and the strains are generally regarded as safe.

Based on the full safety assessment, Mascoma concludes that there is reasonable certainty of no harm to humans consuming beer using the modified *Saccharomyces cerevisiae*. In addition, based on the history of FDA's review of modified *Saccharomyces cerevisiae* for food products under the Generally Recognized As Safe (GRAS) program, Mascoma concludes the manufacturing is in compliance with FDA considerations under the *Food and Drug Cosmetic Act* and accordingly that there is no unreasonable risk of harm to humans or human food.

6.3 Safety of the Donor *Rhizopus oryzae*

R. oryzae is a complex of closely-related, heterothallic species that are widely distributed in nature, prevalent in tropical and subtropical climates, and commonly found in soil, dung and on decaying organic material, such as rotting vegetation. *R. oryzae* is able to grow on a wide range of carbon sources, e.g., glycerol, ethanol, lactic acid, glucose, mannose, fructose, sucrose, xylose, cellobiose, fatty acids, and oils (Maas *et al.*, 2006; Skory, 2000; Yin *et al.*, 1997).

R. oryzae produces a wide range of enzymes that are beneficial to industry, including amylase, glucoamylase, pectinases, cellulases (Saito *et al.*, 2004; Amadioha, 1993; Murashima *et al.*, 2002; Karmakar *et al.*, 2010; Maas *et al.*, 2006). It is able to grow well at a wide temperature range (up to 40 °C) and pH range (from 4 to 9), indicating a robust behavior and widely applicable potential.

R. oryzae is reviewed in the literature as an ancient microbial resource with importance in the modern food industry (Londoño-Hernández *et al.*, 2017). *R. oryzae* strains are often used in Asia for food fermentation to manufacture alcoholic beverages, ragi, or tempeh, and the strains are generally regarded as safe (Meussen *et al.*, 2012; Battaglia *et al.*, 2011; Gryganskyi *et al.*, 2010; Lv *et al.*, 2012). Ragi (ragi tape or tape ketan) is a tapai starter that is a food staple in Indonesia and Malaysia. To make glutinous rice tapai, the rice is first cooked and left to cool. Powdered *ragi* is then mixed into the rice with sugar, wrapped up in banana leaves and allowed to *ferment* for about two days. As expected, fungi, yeast and bacteria grow over this two-day period (Siebenhandl *et al.*, 2001). Lactate formed by enzymes is an essential component of dairy products such as sour milk products, yogurt, ititu (traditional fermented curd), kefir, and cottage cheese. The formation of lactate lowers the pH and promotes curdling of casein in fermented milks; it also contributes to the sour flavor of sourdough breads and is used in beer brewing to lower the pH and add "body" to beer (Simpson *et al.*, 2012). Brewers' amylase produced in powder form from *Aspergillus* species and *Rhizopus oryzae* is used for the manufacture of light beers. Others are Brewers Fermex derived from *Aspergillus* sp. and *Rhizopus oryzae* in powder form for use in increasing the fermentability of wort and cookerzyme derived from *B. subtilis* for accelerated hydrolysis of starch adjuncts (Simpson *et al.*, 2012).

Rhizopus species are also known for producing many traditional foods, such as tempeh, peka, ragi, loog-pang (Londoño-Hernández *et al.*, 2017; Muessen *et al.*, 2012). Strains of the *R. oryzae* complex have been used for centuries as fermented food starters for the production of tempeh and many other Asian foods (Kito *et al.*, 2009; Oda *et al.*, 2003, Ogawa *et al.*, 2004). Chinese yellow rice wine is one of the world's most ancient wines. The microbial diversity of rice wine fermentation starters was evaluated and a variety of filamentous fungi was characterized, including *Rhizopus oryzae* spp., *Mucor spp.*, *Aspergillus* spp., *Penicillium* spp. to name a few. *R. oryzae* was the most frequently detected species in the fermentation starters (Lv *et al.*, 2012).

Rhizopus oryzae is known as an opportunistic human pathogen, however extensive regulatory approvals support the safety of *Rhizopus oryzae* for a multitude of uses. A summary of some of the regulatory activities are as follows:

- FDA has approved the use of carbohydrase derived from *Rhizopus oryzae* for use in the production of dextrose from starch - 21 CFR § 173.130.

- GRN 216 Lipase is produced from *Rhizopus oryzae* for use in the production of dietary triglycerides for supplementing infant formula.
- JECFA evaluated carbohydrase (alpha amylase, pectinase, glucoamylase) from *Rhizopus oryzae* and established an ADI "not limited" in 1972. (http://www.inchem.org/documents/jecfa/jeceval/jec_358.htm, last accessed July 30, 2018).
- In Canada, Table V – food additives that may be used as food enzymes – provides a list of enzymes, sources, and uses. *Rhizopus oryzae* is used as a permitted source for the production of amylase, glucoamylase, lipase, and pectinase that may be used in the production of ale, beer, light beer, malt liquor, porter, stout, cider, wine, and cheese.

As mentioned previously, the lactate dehydrogenase enzyme gene from *Rhizopus oryzae* was amplified by PCR from an artificially synthesized gene based on the Genbank sequence, which negates the possibility of donor DNA transfer to the strain.

In conclusion, *Rhizopus oryzae* has a history of use food applications without adverse effects and is a suitable gene donor for our modified *Saccharoymces cerevisiae* for use in beer production.

6.4 Safety of the Lactate Dehydrogenase Enzyme

LDH can make pyruvate from lactate or lactate from pyruvate, as it converts NAD+ to NADH and back. The conversion of lactate to pyruvate is an important step in energy production in cells. LDH exists in nearly all living cells and is relatively rich in mammalian tissues, such as heart, kidney, liver, muscle and blood. The presence of LDH was discovered in human blood serum in 1954 and LDH is released into the bloodstream as red blood cells die (Danese *et al.*, 2016). LDH isozymes have been reported in the developing fetus and children have elevated LDH production with bone growth.

LDH is of interest clinically as a biomarker that can be measured in the serum. Levels of certain LDH isozymes may reflect pathological conditions in particular tissues and/or diseases (Miao *et al.*, 2013). As a widely researched enzyme, the literature also reports many other findings such as that LDH activity drives hair follicle stem cell activation (Aimee *et al.*, 2017).

LDH is present in most cells of the human body, mainly concentrated in heart, liver, RBCs, kidneys, muscles, brains and lungs. Elevated serum LDH levels (> 600 IU/l) is typically associated with an abnormal physiological condition or disease state (Vasavi kadiyala, 2015; Koukourakis *et al.*, 2008; Yu *et al.*, 2014). Cancer cells are associated with enhanced lactate production due to increased glycolytic activity that correlates with high glucose uptake, regardless of oxygen availability. Known as the Warburg effect, this phenomenon describes the ability of tumor cells to adapt to its microenvironment to meet its energy needs. This tumor environment is highly hypoxic, which means that tumor cells have an intensified anaerobic metabolism. Six isozymes are known, but it is LDH-5 that is a key player in the Warburg effect, which catalyzes the formation of lactate in the final step of the glycolytic pathway. Studies have confirmed that LDH-5 plays a crucial role in tumor maintenance and that elevated LDHA gene expression characterizes many human tumors.

LDH is widely distributed in nature being found in vertebrates, plants and bacteria (Tsuji *et al.*, 1994). Fruits and vegetables such as avocado, pear, lettuce, and strawberry, meat and fermented foods contain LDH (Ke *et al.*, 1993; Oba *et al.*, 1977; Kato-Noguchi, 1998; Collins *et al.*, 1991).

The Enzyme Technical Association (ETA) assessed the safety of orally administered enzyme food supplements and concluded that given "the long history and common used in food, efficacy testing is not required to demonstrate the safety of enzymes. Microbially (which includes fungal) sourced enzymes have been the subject of significant safety and toxicity testing for their use as direct additives and processing aids in the food (and feed) industries. The safety of enzyme supplements should be determined by the history of safe use and the accumulated safety and toxicity data that have been generated over the last 40 years for food-uses of the same enzymes, as well as the international recognition of food enzymes as being intrinsically safe proteins" (ETA, 2012).

Several groups have demonstrated efficient production of L-lactic acid from *Saccharomyces cerevisiae* expressing exogenous genes encoding LDH. In particular, the literature shows examples of the production of lactic acid and ethanol from engineered *Saccharomyces cerevisiae* containing a LDH gene from *R. oryzae (*Skory, 2003, 2004; Ishida *et al.*, 2005; Dequin & Barre, 1994; Porro *et al.*, 1995). One such example shows that an engineered yeast strain produced high LDH activity and produced lactic acid from glucose or xylose. Ethanol was the major fermentation product and lactic acid was the minor product when engineered yeast was grown on glucose (Turner *et al.*, 2015).

As referenced above, there are numerous processes in which *R. oryzae* is used in food production (Londoño-Hernández *et al.*, 2017; Muessen *et al.*, 2012), with attributable levels of lactic acid. Whereas no studies have been performed specifically identifying the lactate dehydrogenase present during *R. oryzae* food fermentations, one can infer the presence of the enzyme as it has been shown to be the primary enzyme responsible for lactic acid production. Skory (2000) specifically isolated and sequenced the *R. oryzae* LDH of interest during anaerobic growth on pure sugars. Furthermore, the author also cloned the LDH gene and complemented its functionality into *E.coli*, further confirming that this protein is the primary enzyme responsible for lactic acid production during a *R. oryzae* fermentation. In a subsequent study, the same author increased lactic acid production in *R. oryzae* by introducing additional copies of the LDH sequence (Skory, 2004). Similarly, Skory and Ibrahim (2007) complemented the *R.oryzae* LDH gene into a fumaric acid producing isolate of *R. oryzae* which was deficient in lactic production, and consequently increased lactic production to over 27g/L. Gheinani et al (2011), used RNA silencing to target the LDH gene and demonstrated an 85% reduction in overall lactic production, further confirming the LDH sequence as the primary lactic producer.

In conclusion, we were unable to identify any risk factors for the expression of lactate dehydrogenase from *Rhizopus oryzae* in *Saccharomyces cerevisiae* for beer production.

6.4.1 Allergenicity

Enzymes are typically used as processing aids and have a long history of safe use in food, with no indication of adverse effects or reactions (Pariza & Foster, 1983). In 1998, the Working Group on Consumer Allergy Risk from Enzyme Residues in Food of the Association of Manufacturers of Fermentation Enzyme Products (AMFEP) conducted an in-depth analysis of

the allergenicity of enzyme products. The study concluded that there are no scientific indications that small amounts of enzymes in bread and other foods can sensitize or induce allergy reactions in consumers and concluded that enzyme residue in bread and other foods do not represent any unacceptable risk to consumers. Exposure to enzymes via food is almost always low; generally, enzymes are added at the lowest level concentrations (parts per million) to obtain its reaction necessary for its application.

In addition, the enzyme is typically removed or denatured during food processing and denatured protein has been shown to be very susceptible to digestion in the gastro-intestinal system. A wide range of naturally-occurring food enzymes have been shown to be very labile in the gastro-intestinal system even in native unprocessed form.

According to the literature, the majority of proteins are not allergens; only 0.3% of all identified proteins are listed as allergens. A wide variety of enzyme classes and structures are naturally present in plant and animal-based foods. Based on enzymes long history of safe use in the production of foods, food enzymes are not homologous to known allergens and enzymes such as lactate dehydrogenase with a history of safe use have not raised safety concerns for food allergies (Bindslev-Jensen *et al.*, 2006).

Despite the general lack of concern for allergies by enzymes, potential allergenicity of the lactate dehydrogenase protein was evaluated using the full length FASTA and sliding 80 amino acid segments. The history of using exact 8 amino acid matching algorithms has indicated that the method is not predictive and is generally discounted as an approach to evaluating allergenicity. Therefore, this analysis should not be used (Ladics *et al.*, 2011; AllergenOnline, available at http://www.allergenonline.org/).

The protein's amino acid sequence was compared against known allergens using the Food Allergy Research and Resource Program (FARRP) Protein AllergenOnline Database (version 18B; released March 23, 2018; available at http://www.allergenonline.org/). This database includes a comprehensive list of putative allergenic proteins developed via a peer-reviewed process for the purpose of evaluating food safety. The *Rhizopus oryzae* lactate dehydrogenase amino acid protein sequence expressed in *Saccharomyce cerevisae* is provided in **Appendix 1**.

In accordance with the guidelines endorsed by Codex Alimentarius Commission (2009) and EFSA (2010) for the safety evaluation of newly expressed proteins from genetically modified plants and microorganisms, the database (AllergenOnline Database, version 18B; http://www.allergenonline.org/) was searched using a sliding window of 80-amino acid sequences derived from the full-length amino acid sequences. According to the approach adopted by the Codex Alimentarius Commission, significant homology is defined as an identity match of greater than 35%, and in such instances, cross-reactivity with the known allergen should be considered a possibility. The 35% identity for 80 amino acid segments is a suggested guideline proposed by the Codex Alimentarius Commission for evaluating newly expressed proteins produced by recombinant-DNA plants (2009).

The sequence homology search was performed (AllergenOnline, v18A, February 1, 2018) and no identity matches of greater than 35% were identified for the sequence evaluated. Based on these

search results, no evidence exists that suggests that the expressed protein would cross-react with known allergens.

According to recent analysis, FASTA or BLASTP searches may be the most predictive approach for allergeric reactions (Aalberse, 2000; Goodman & Teeteh, 2011; Goodman *et al.*, 2016) and according to Ladics *et al.* (2007) "resulted in identity matches that better reflected functional similarities between proteins." Ladics *et al.* (2011) suggests using the 35% threshold or greater shared amino acid sequence using this method. Using the FASTA alignment of the amino acid protein sequence with known allergens using the AllergenOnline Database (version 18B; http://www.allergenonline.org/) did not result in alignment with allergenic proteins at or above the 35% threshold of concern for allergenicity.

As indicated above, enzymes are unlikely to be food allergens. In addition, the enzyme is typically removed or denatured during beer production. Therefore, it's concluded that the expressed lactate dehydrogenase enzyme encoded in the *Saccharomyces cerevisiae* is unlikely to be a concern with regard to food allergy.

6.4.2 Safety Assessment Based on Decision Tree Analysis

An evaluation of the modified *Saccharomyces cerevisiae* strain based on criteria set forth by experts (Pariza & Foster, 1983; IFBC, 1990; EU SCF, 1991; OECD, 1992; FAO/WHO, 1996; Pariza & Johnson, 2001) demonstrates the safety of these genetically modified production strain. This evaluation includes the identity of the host strain, a description of the introduced DNA (the sources and functions of the introduced genetic material), an outline of the genetic construction of the production strain, and a characterization of the production strain.

Pariza and Foster base the decision tree concept on their 1983 publication that focused on the safety evaluation methodology of enzymes used in food processing, which was extended further by the International Food Biotechnology Council into the decision tree format (IFBC, 1990). In 2001, Pariza and Johnson published updated safety guidelines further building on the IFBC and other reports (Kessler et al., 1992) including considerations using rDNA technologies. The literature emphasizes that production strain safety is the primary consideration in evaluating enzymes derived from microorganisms, with particular focus on the toxigenic potential of the production strain. More specifically, the authors elaborate on the *safe strain lineage* concept and the elements critical to establish the safety of a production strain. "Thoroughly characterized non-pathogenic, non-toxigenic microbial strains, particularly those with a history of safe use in food enzyme manufacture, are logical candidates for generating safe strain lineage, through which improved strains may be derived via genetic modification by using either traditional/classical or rDNA strain improvement technologies." (Pariza & Foster, 1983). To establish safe strain lineage, the decision tree addresses elements such as "thoroughly characterizing the host organism, determining the safety of all new DNA that has been introduced into the host organism, and ensuring that the procedure(s) that have been used to modify the host organism are appropriate for food use" (Pariza & Johnson, 2001).

Pariza and Johnson (2001) outline a twelve-step decision tree for determining the safety of the production strain. In particular, by answering specific questions set forth in the decision tree, including whether the strain is non-pathogenic, free of antibiotics, and free of oral toxins (or

below limits of concern), the production strain can be accepted as derived from a safe lineage at step 6 or step 11. Otherwise, step 12 concludes that there may be "an undesirable trait or substance" present and the production strain may be 'unacceptable' in step 13. If the "genetic potential for producing the undesirable trait or substance can be permanently inactivated or deleted," the decision tree suggests that the "test material may be passed though the decision tree again."

Mascoma's decision tree analysis, based on the 2001 decision tree, is shown in **Appendix 2**. The production strain is genetically modified using standard recombinant DNA techniques, and the gene is integrated into a designated locus of the *Saccharomyces cerevisiae* parental strain. The production strain is free of transferable antibiotic resistance gene DNA. The introduced DNA is well-characterized and free of attributes that would render it unsafe for potable beer production.

6.5 Reports or Investigations Which May Appear to Be Inconsistent with the GRAS Conclusion

6.5.1 Discussion of scientific literature that claims Saccharomyces cerevisiae is a pathogen in immunocompromised individuals

The literature reports that *S. cerevisiae* is an opportunistic pathogen. An extensive literature search on the safety of *Saccharomyces cerevisiae* reveals that for over the last fifty years, there have been reported cases of infections in mostly immunocompromised individuals (Eschete *et al.*, 1980; Eng *et al.*, 1984; Hazen, 1995; Murphy & Kavanagh, 1999; EFSA, 2008). McCusker (2006) provides a list of *S. cerevisiae* infections described in the literature. While the list includes infections in patients with AIDS; it does not identify which of the other patients were otherwise immunocompromised. Additionally, in a review of reported cases of invasive *S. cerevisiae* and *Saccharomyces boulardii* fungemia, Enache-Angoulvant *et al.* (2005), identified 92 reports, 76 of which were diagnosed between 1990 and 2005. These cases were frequently nosocomial in origin, primarily associated with central intravenous catheter (CVC) use or previous antibiotic therapy and each patient exhibited at least one underlying condition that might expedite the development of an invasive fungal infection.

Muñoz *et al.*, (2005) described 3 intensive care unit patients that had *S. cerevisiae* fungemia at Hospital General Universitario. As part of the report, the authors searched MEDLINE for reports of *S. cerevisiae* fungemia since 1966. Their search returned only 57 additional reported cases.

Since *S. cerevisiae* is commonly used in the biotechnology industry, Murphy and Kavanagh (1999) also examined its potential pathogenicity. They also concluded that *S. cerevisiae* can be regarded as an opportunistic pathogen for the immunocompromised, but one of low virulence.

Saccharomyces cerevisiae fungemia has been seen to manifest as unexplained fever, pneumonia, esophagitis, empyema, liver abscess, peritonitis, vaginitis, urinary tract infection, cellulitis, or septic shock (Lherm *et al.*, 2002; Williams *et al.*, 2007; Pfaller & Diekema, 2010; Kliemann *et al.*, 2011). A rare case was reported where a baker exhibited evidence of a *S. cerevisiae* induced

lung nodule (Ren *et al.*, 2004), indicating that *S. cerevisiae* has some potential to colonise following inhalation exposure. However, even this route will carry a much greater risk in individuals with pre-existing medical conditions that might predispose them to fungemia, such as hospital residents (Kelesidis & Pothoulakis, 2012). It is generally recognized that the main entry points for *Saccharomyces cerevisiae* into the blood stream are enteral translocation following antibiotic induced yeast overgrowth or CVC hub/insertion site contamination (Enache-Angoulvant *et al.*, 2005; Pfaller & Diekema, 2010).

Despite these rare opportunistic infections, the FDA (and NIH), EPA, and EFSA maintain the safety of *Saccharomyces cerevisiae* as a nonpathogenic microorganism. EFSA notes that "[r]are opportunistic infections have been caused by *S. cerevisiae*," and EFSA maintains its QPS (Qualified Presumption as Safe) status (EFSA, 2008, p.27; EFSA, 2013, p.23). EFSA provides additional clarification stating, "*S. cerevisiae*, subtype boulardii is contraindicated for patients of fragile health, as well as patients with a central venous catheter in place. A specific protocol concerning the use of probiotics should be formulated" (EFSA, 2008, Table 4, pp.21, 43). Even with the infrequent cases of fungemia associated with *S. boulardii*, McFarland (2010) discusses contraindications and precautions and recommends closely monitoring adult immunocompromised patients and catheter use, especially with unexplained fever and notes that some recommend not giving *S.boulardii* to immunosuppressed patients or those with central catheters to reduce the risk of fungemia (Buts, 2009).

As EPA recognized in its Final Risk Assessment of *Saccharomyces cerevisiae* (February 1997) (p.9), "[m]any scientists believe that under appropriate conditions any microorganism could serve as an opportunistic pathogen." The Agency concluded that *Saccharomyces cerevisiae* has an extensive history in food processing and neither it nor other closely related species "has been associated with pathogenicity toward humans or has been shown to have adverse effects on the environment" (p.2).

6.5.2 Discussion of scientific literature that claims Rhizopus oryzae is a pathogen in immunocompromised individuals

Rhizopus oryzae is known as an opportunistic human pathogen and has a high prevalence under mucormycosis infections (Roden *et al.*, 2005). According to the CDC, mucormycosis (previously called zygomycosis) is a serious but rare fungal infection caused by a group of molds called mucormycetes. These molds live throughout the environment. Mucormycosis mainly affects people with weakened immune systems and can occur in nearly any part of the body. It most commonly affects the sinuses or the lungs after inhaling fungal spores from the air, or the skin after the fungus enters the skin through a cut, burn, or other type of skin trauma (www.cdc.gov/fungal/diseases/mucormycosis/index.html, last accessed July 30, 2018). Most mucormycosis cases have an underlying illness such as an elevated serum iron level, trauma, or a weakened immune system (Royer & Puéchal, 2014; Ibrahim, 2011; Roden *et al.*, 2005).

Fungal allergy is considered as serious health problem worldwide. *Rhizopus oryzae* is a ubiquitously present airborne pathogenic mold and an important source of inhalant allergens for the atopic population of India (Sircar *et al.*, 2015). *Rhizopus* species can also act as

opportunistic, invasive animal and human pathogens that cause deadly infections in immunocompromised individuals.

Based on the regulatory and safety reviews, *Rhizopus orzyae* is recognized as an acceptable donor and organism for the production of a variety of enzymes for use in food production. Although there is very limited safety and toxicological studies published, strains of *Rhizopus oryzae* have been reviewed by FDA and other regulatory authorities for use as a production organism for a wide range of commercially relevant enzymes that are used as processing aids in the food industry. *Rhizopus oryzae* strains have been used for centuries in the production of Asian foods (such as alcoholic beverages, ragi, or tempeh). The production of L-(+) lactic acid by *R. oryzae* and other production organisms is used in the food and animal feed industries (Goldberg *et al.*, 2006; Datta & Henry, 2006). In addition, special considerations have been established for a few fungi like *R. oryzae* that have a long history of safe use in the food industry (Pariza & Foster, 1983). Therefore, *Rhizopus oryzae* poses no known safety risk as the donor organism.

6.6 Conclusions for GRAS Determination

The following conclusions are made for modified *Saccharomyces cerevisiae* with lactate dehydrogenase from *Rhizopus oryzae* for use as a processing aid to manufacture alcoholic beer:

- A review of the published literature shows a long history of safe use of *Saccharomyces cerevisiae*, commonly known as bakers or brewer's yeast, for thousands of years of use in alcohol, brewing and baking. Individually, both *Saccharomyces cerevisiae* and *Saccharomyces cerevisiae*-derived products are approved food additives, affirmed as GRAS substances, and the subject of previous GRAS Notifications.

- The modified *Saccharomyces cerevisiae* strain is derived from a native *Saccharomyces cerevisiae* yeast that is used in the brewing industry. The production strain has been determined to be substantially equivalent to the host strain with respect to overall performance such as growth rate, fermentation rate and ethanol production.

- The modified *Saccharomyces cerevisiae* strain is constructed via linear DNA transformation with synthetic genes to avoid any unintended transfer of genetic elements from the donor strain to the host strain. Thus, the modified yeast contains only a limited introduced sequence pertaining to the gene of interest. Furthermore, the LDH donor organism *Rhizopus oryzae* has a safe history of use in food and we were unable to identify any risk factors for using *Rhizopus oryzae* as a gene donor.

- The LDH enzyme exists in nearly all living cells, including human cells, has been studied extensively and we were unable to identify any risk factors for the expression of lactate dehydrogenase from *Rhizopus oryzae* in *Saccharomyces cerevisiae* for beer production.

- The lactate dehydrogenase enzyme in the modified *S. cerevisiae* produces lactic acid during fermentation as flavoring in beer production. Lactic acid is considered GRAS as a

direct food ingredient when used in food as a flavoring agent with no limitation other than current good manufacturing practice.

- The modified *Saccharomyces cerevisiae* strain is produced according to the principles of GMP, using food-grade ingredients or ingredients that are acceptable for general use in foods as specified under FCC guidelines. Physical inspection and the appropriate chemical and microbiological analyses are conducted to confirm strain identity, no contamination, and to ensure the yeast product meets the specifications set forth in Section 2.4.

- The modified *Saccharomyces cerevisiae* production strain was determined to meet the safe strain criteria, based on the decision tree analysis developed by Pariza and Johnson (2001) for evaluating the safety of microbial enzymes.

- Limited to no viable amounts of modified *Saccharomyces cerevisiae* remains in the beer products after pasteurization and/or filtration, both of which are standard practice in the brewing industry.

The modified *Saccharomyces cerevisiae* product should be regarded as substantially equivalent to the parent yeast strain in terms of its safety, utility and functionality, with the exception of its ability to produce lactate dehydrogenase. Based on this evaluation and a review of the scientific literature, it is concluded that the modified *Saccharomyces cerevisiae* with lactate dehydrogenase from *Rhizopus oryzae* is GRAS for use in the production of potable alcoholic beer and exempt from the premarket approval requirements.

7. List of Supporting Data and Information

Appendix 1: The Amino Acid Sequence of the Lactate Dehydrogenase
Appendix 2: Safety Decision Tree

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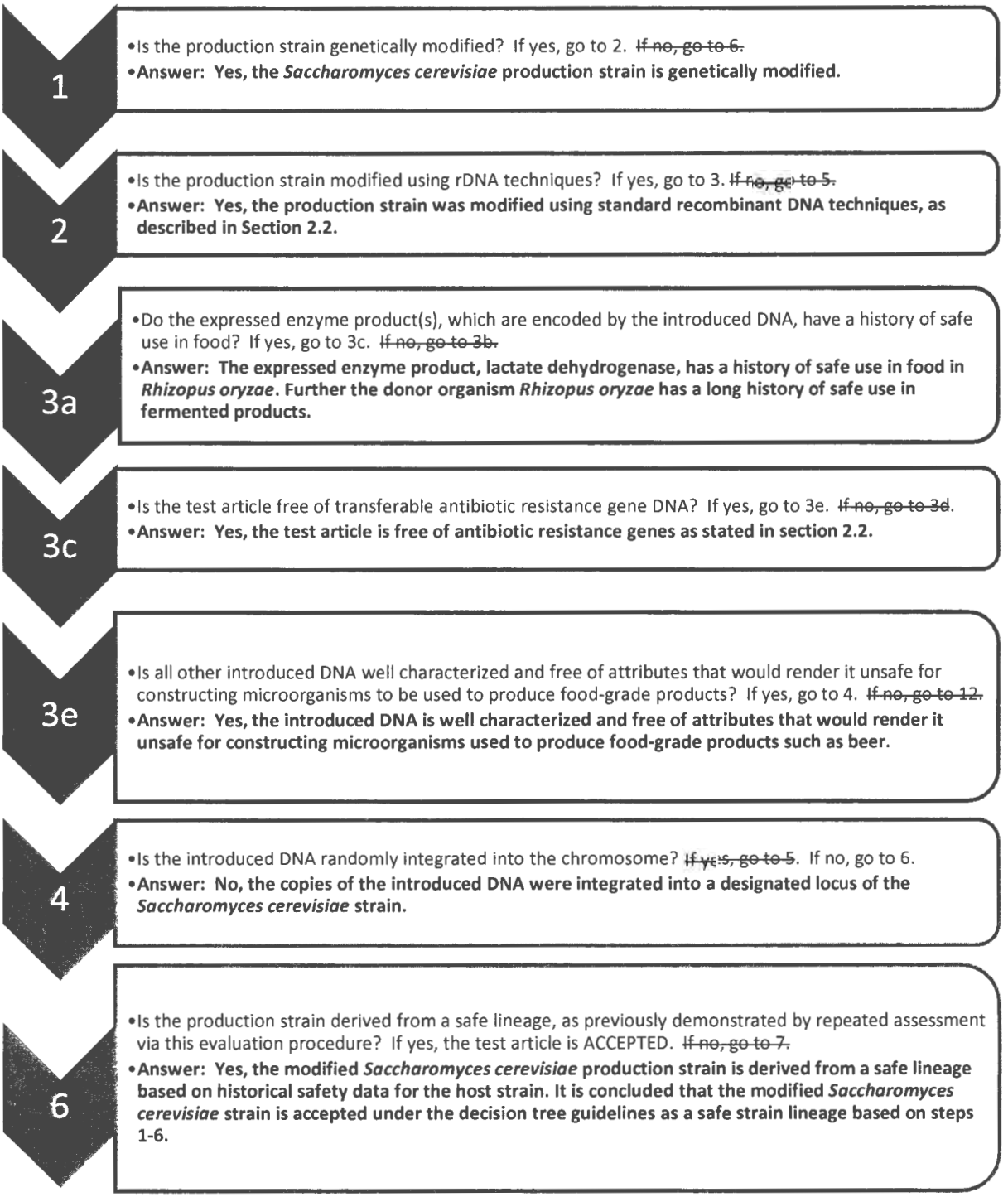
APPENDIX 1: The Amino Acid Sequence of the Lactate Dehydrogenase

The strain expresses two copies of the lactate dehydrogenase gene. This gene was designed by creating a synthetic DNA sequence (codon optimized for *Saccharomyces cerevisiae*) based on the amino acid sequence of the non- modified wild-type lactate dehydrogenase from *Rhizopus oryzae* (Table A2-1), therefore neither the donor organism nor its DNA was actually used to modify the production organism yeast.

Table A2-1. Native amino acid sequence for lactate dehydrogenase from *Rhizopus oryzae*

Gene	Native Sequence
Lactate dehydrogenase	MVLHSKVAIVGAGAVGASTAYALMFKNICTEIIVVDVNPDIVQAQVLDLA DAASISHTPIRAGSVEEAGQADIVVITAGAKQREGEPRTKLIERNYRVLQSII GGMQPIRPDAVILVVANPVDILTHIAKTLSGLPPNQVIGSGTYLDTTRLRVH LGDVFDVNPQSIHAFVLGEHGDSQMIAWEAASIGGQPLTSFPEFAKLDKTA ISKAISGKAMEIIRLKGATFYGIGACAADLVHTIMLNRKSVHPVSVYVEKY GATFSMPAKLGWRGVEQIYEVPLTEEEEALLVKSVEALKSVEYSSTKVPEK KVHATSFSKSNC

APPENDIX 2: Safety Decision Tree for Modified *Saccharomyces cerevisiae*



Conclusion: ACCEPTED, under Decision Tree Guidelines

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
000841	
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)*: 2018-09-18

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)*: _____

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SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position or Title
Joanne Donoghue	Director, EHS & Operations
Organization *(if applicable)*	
Mascoma LLC	
Mailing Address *(number and street)*	
67 Etna Road, Suite 200	

City	State or Province	Zip Code/Postal Code	Country
Lebanon	New Hampshire	03766	United States of America

Telephone Number	Fax Number	E-Mail Address
603-676-3320		jdonoghue@mascoma.com

1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position or Title
Martha Marrapese	Attorney at Law
Organization *(if applicable)*	
Wiley Rein LLP	
Mailing Address *(number and street)*	
1776 K Street NW	

RECEIVED JAN 29 2019 OFFICE OF FOOD ADDITIVE SAFETY

City	State or Province	Zip Code/Postal Code	Country
Washington	District of Columbia	20006	United States of America

Telephone Number	Fax Number	E-Mail Address
202-719-7156		mmarrapese@wileyrein.com

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

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

Saccharomyces cerevisiae with lactate dehydrogenase

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? *(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 744

☐ 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)* GRN 626, 422, 350, 175, 120, 88, and 216

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.

The modified Saccharomyces cerevisiae is used as a processing aid in the fermentation of alcoholic beer. The yeast with the lactate hydrogenase enzyme is used in brewing at levels to achieve the desired effects for alcohol and lactic acid content (sourness) according to good manufacturing practices. The recommended pitching rate of the yeast is 50 - 100 g/hL to achieve a minimum of 2.5 to 5 million cells/mL at the beginning of the beer fermentation. The subpopulation expected to consume the substance are men and women over the age of 21 due to the legal drinking age in the United States.

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 Mascoma LLC
(name of notifier)

has concluded that the intended use(s) of Saccharomyces cerevisiae with lactate dehydrogenase
(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. Mascoma 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.

67 Etna Road, Suite 200, Lebanon, NH 03766
(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)
jdonoghue@lallemand.com Digitally signed by jdonoghue@lallemand.com Date: 2019.01.16 13:34:20 -05'00'	Joanne Donoghue, DIrector EHS & Operations	01/16/2019

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)
	GRASNotice_ScerevisiaewithLDH_ 2019-01-16.pdf	Submission
	Appendix1AminoAcidSequence_ScerevisiaewithLDH_2019-01-16.pdf	Submission
	Appendix2SafetyDecisionTree_ScerevisiaewithLDH_2019-01-16.pdf	Submission

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.