GRAS Conclusion

A lipase from *Fusarium oxysporum* produced by *Saccharomyces cerevisiae*

is Generally Recognized As Safe

for Use in Baking

December 21, 2021

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1. Signed Statements and Certifications

1.1 Exemption from Premarket Approval

Lallemand Inc. has determined that its lipase enzyme produced by *Saccharomyces cerevisiae* expressing the gene encoding a sequence of lipase from *Fusarium oxysporum* is a Generally Recognized as Safe ("GRAS") substance for the intended food application and is, therefore, exempt from the requirement for premarket approval.

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

Lallemand inc.
1620 Prefontaine Street
H1W 2N8, Montreal, QC, Canada

1.4 Common Name of the Notified Substance

Lipase (synonym triacylglycerol lipase) food enzyme, produced from *Saccharomyces cerevisiae* expressing lipase from *Fusarium oxysporum*.

1.5 Intended Conditions of Use

The lipase enzyme is to be used in baking processes. The lipase enzyme will be denatured during the baking process and will be present in insignificant quantities as inactive residue. This product is intended to replace other lipases currently in commercial use for this application that are produced in other microorganisms, including *Aspergillus oryzae* and *Trichoderma reesei.*

1.6 Availability of Information for FDA Review

A notification package providing a summary of the information that supports this GRAS conclusion is enclosed with this notice. 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 during customary business hours at 1620 Prefontaine Street, H1W 2N8, Montreal, QC, Canada or will be sent to the Food and Drug Administration upon request.

Please direct all inquiries regarding this GRAS determination to:

Celia Martin
cmartin@lallemand.com

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.

Lallemand Inc. 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

December 21, 2021

Celia Martin, PhD — Date
Regulatory Affairs Director
Lallemand Inc.

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

2.1 Identity of the Notified Substance

The subject of this notification is a lipase produced by fermentation of a genetically modified *Saccharomyces cerevisiae* strain expressing the gene encoding a lipase from *Fusarium oxysporum*.

IUB Name: Triacylglycerol lipase

Systematic name: Triacylglycerol acylhydrolase

Other name(s): Lipase, triglyceride lipase, glycerol ester hydrolase, tributyrase, butyrinase, tributyrinase, tributyrin esterase, triglyceride hydrolase; triglyceridase; triacylglycerol ester hydrolase

IUBMB Number: EC 3.1.1.3

CAS registry number: 160611-47-2

Reaction: Triacylglycerol lipase or lipase (EC 3.1.1.3) catalyzes the hydrolysis of triglycerides ester bonds into diglycerides and subsequently into monoglycerides and glycerol, as well as free fatty acids.

Production strain: *Saccharomyces cerevisiae* LALL-LI

Amino acid sequence: The total nucleotide and amino acid sequences have been determined.

2.2 Identity of the Source

2.2.1 Production Strain

The production organism LALL-LI is a strain of *Saccharomyces cerevisiae* that has been genetically modified to express a lipase gene that is native to *Fusarium oxysporum*. The gene was amplified by polymerase chain reaction (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, 2006).

The production strain has been confirmed to be *Saccharomyces cerevisiae* by whole genome sequencing and phylogenetic analysis.

Taxonomic characteristics of the production strain:

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

2.2.2 Host Microorganism

The *Saccharomyces cerevisiae* host strain yeast is a baker's yeast strain (with no prior genetic modifications) and is similar to other commercial baking strains.

2.2.3 Lipase from Fusarium Oxysporum

No material from the donor organism was used in the construction of the modified strain, to prevent any carryover of donor strain genetic material when engineering our yeast strain. Therefore, no DNA from the donor organism is present in the final strain.

Using an amino acid sequence of *F. oxysporum* lipase that is publicly available on Genbank (excluding the first 15 amino acids as the first 15 amino acids are predicted to be the signal peptide, which would not be present in the mature protein[1]), a DNA sequence codon-optimized for S. cerevisiae was synthesized, which allows for efficient expression in the host strain without introducing further changes to the amino acid sequence of the heterologous protein.
To the DNA sequence coding for the lipase enzyme, the DNA sequence encoding a hybrid signal peptide was added to the 5' end of the DNA sequence coding for the lipase, to facilitate efficient secretion by *S. cerevisiae*. The signal peptide is to be cleaved during cellular processing of enzymes destined for secretion, leaving behind the mature peptide, which is the lipase sequence.

2.2.4 Construction of the Production Strain

The molecular tools and practices used during the construction of the production strains are standard to the field of biotechnology and yeast genetics. The genetic modification techniques utilized to develop these modified strains relies upon directed integration to insert the genes at specific and known sites 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 lipase expression cassette was stably integrated into the *S. cerevisiae* genome at a specific locus using homologous recombination, a very efficient process in *S. cerevisiae*. This was done using a

[1] Signal peptide prediction was performed using SignalP-5.0 server http://www.cbs.dtu.dk/services/SignalP-5.0

method that enables one-step integration into the yeast genome without needing to integrate antibiotic resistance markers into the genome, under the regulation of native S. cerevisiae promoters and terminators.

Furthermore, in order to facilitate correct folding of the lipase protein through the secretory pathway, the strain was further engineered to obtain increased expression levels of two *S. cerevisiae* native chaperones. For both chaperones the DNA was directly amplified from the host strain *S. cerevisiae* (unmodified parent strain) genomic DNA to achieve identical sequences as the wild-type yeast strain. The overexpression cassette of the chaperones was integrated using the same method as the lipase expression cassette, also at a specific locus, under the regulation of native S. cerevisiae promoters and terminators.

The genetic construction was confirmed by PCR analyses and phenotypic characterizations. 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 lipase sequence and the promoters and terminators.
PCR genotyping and whole genome sequencing confirmed that the inserts were integrated into the yeast genome at the intended loci. The production strain has multiple copies of lipase present in the genome.

2.2.5 Stability of the 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.

To determine the genotypic stability of the production strain throughout the propagation procedure, genomic DNA was isolated from the cells used for seeding the yeast propagation (slant), and also from the final cream yeast at the end of the yeast propagation. The isolated genomic DNA was used for PCR genotyping to confirm the genotypic stability of the strain. PCR genotyping shows that both populations show the same genetic pattern across the recombinant DNA cassette. Furthermore, quantitative PCR shows stability of lipase gene copy number from a slant to yeast cream. It has also been confirmed by PCR that the expression cassette for the two native *S. cerevisiae* chaperones is also stable throughout the propagation.

2.2.6 Antibiotic Resistance Genes

During construction of the engineered strain, only a single plasmid was used during the transformation step, which contains the hygromycin resistance gene. This plasmid was only used as a co-transformation aid and no plasmid DNA was integrated into the yeast genome. The plasmid was cured with passaging of the transformant on non-selective media. Absence of antibiotic markers was confirmed by whole genome sequencing and by assessing growth on selective media.

Therefore, confirmation of removal of any antibiotic resistance genes was confirmed and no antibiotic resistance was conferred to the modified strain.

2.2.7 Absence of the Production Organism in the Final Product

The absence of the production strain in the final product is an established specification for the commercial product. Therefore, the production organism does not end up in food.

2.3 Method of Manufacture

The lipase enzyme preparation described herein is produced by fermentation of *Saccharomyces cerevisiae* LALL-LI, followed by recovery (downstream processing), formulation and packaging. It is produced in accordance with current good manufacturing practices for food (cGMP). When production is run in the EU, it is also subject to the Food Hygiene Regulation (852/2004).
The manufacturing flow-chart is presented in Appendix 1.

2.3.1 Manufacturing in the Enzyme Production Plant

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 production facilities with established procedures and equipment suitable for Good Industrial Large-Scale Practice (GISLP) and meets the criteria for safe production organism as described in Pariza and Johnson (2001).
Physical inspection and the appropriate microbiological and fermentation analyses are conducted to confirm strain identity and functionality in application, ensuring that the 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 & Whitaker, 1995).

The culture stocks are sent to the yeast plant (as frozen vials or as slants) from the location of the master cell bank. The plant keeps a record of all stocks received and used in production. A unique sequential number is assigned to each stock to ensure traceability during all steps of production. During production, many parameters are checked according to the Quality Plans and Inspection Plans in place. Inspection Plans are developed to ensure testing during critical steps of the production process from beginning to end. Many parameters are followed such as physical-chemical analysis (solids, color, pH, *etc*.), microbiological analysis and processing activities.

2.3.2 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, a nitrogen source, other nutrients (essential elements and vitamins), pH adjustment agents and foam control agents. For the recovery process, filter-aids, pH adjustment agents, foam control agents and flocculants might be used.

The raw materials conform to either specifications set out in the Food Chemical Codex or The Council Regulation 93/315/EEC, setting the basic principles of EU legislation on contaminants and food, and Commission Regulation (EC) No 1881/2006 setting maximum limits for certain contaminants in food. 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.

2.3.3 Fermentation at Laboratory Stage

Yeast propagation is initiated from frozen master stocks of pure culture maintained at -80°C in glycerol. The strain may be struck from the master cell bank to a sterile agar slant, and the slant may be used to inoculate a flask of 5-10L of sterile medium (autoclaved) under strict sterile conditions. Alternatively, 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.

2.3.4 Fermentation at Plant Stage

The yeast from the flask is inoculated into a propagation tank. The culture is then sequentially transferred into increasing fermenter volumes and the fermentation process is continued for a predetermined time.

To prevent contamination of foreign microorganisms, all equipment is carefully operated, cleaned, and maintained. 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 feeding rate of carbon source is adjusted to provide the optimal growth with minimal ethanol production.

2.3.5 Recovery and Formulation of the Finished Product

The recovery process is initiated upon completion of fermentation. During fermentation, the enzyme protein is excreted by the producing strain into the fermentation medium.

The recovery process is a multi-step operation designed to separate the enzyme from the microbial biomass and partially purify and concentrate the enzyme.

The enzyme is recovered from the culture broth by the following series of operations:

1) Primary solid/liquid separation – Filtration or centrifugation.

2) Polish filtration - for removal of residual production strain organisms and as a general precaution against microbial degradation.

4) Ultrafiltration – For concentration and purification.

The nature, number, and sequence of the different types of unit operations may vary, depending on the specific enzyme production plant.

Subsequently, the enzyme is formulated. Lipase enzyme is sold mainly as a solid preparation, but can also be sold as a liquid preparation, after addition of stabilizing and preservation agents, including, but not limited to sucrose, glycerol, sodium chloride, potassium sorbate and sodium benzoate.

Drying can be done using various technologies in order to deliver the preferred particle properties. Carriers, typically salt, starch or dextrin, can be added to improve the drying process. All carriers are GRAS. The food enzyme is adjusted to a declared activity.

The food enzyme preparation is tested by Quality Control for all quality related aspects, like expected enzyme activity and the general JECFA Specification for Food Enzyme Preparations (JECFA, 2006) and released by Quality Assurance.
The final product is packed in suitable food packaging material before storage. Warehousing and transportation are performed according to specified conditions mentioned on the accordant product label for food enzyme preparations.

2.3.6 General Production Controls

To confirm that the manufactured food enzyme preparation is of food-grade quality and meets international standards/specifications for food enzymes, the food enzyme is analyzed for potential impurities and contaminants that may originate from the production strain or manufacturing process, and complies with the general JECFA specifications for food enzyme preparations (JECFA, 2006).

To ensure that the food enzyme preparation meets these quality criteria, potential hazards are taken into account and controlled during the whole production process as described below:

i) Microbiological Hygiene

For optimal and qualitative enzyme production, it is important that hygienic conditions are maintained throughout the entire fermentation process. Actions in place to guarantee microbiological hygiene and prevent contamination with microorganisms ubiquitously present in the environment (water, air, raw materials) are as follows:

• Hygienic design of equipment: All equipment is designed, constructed and used to prevent contamination by foreign micro-organisms.

• Cleaning and sterilization:

  o Validated standard cleaning and sterilization procedures of the production area and equipment: all fermenters, vessels and pipelines are washed after use with a CIP-system (Cleaning in Place). After cleaning, the vessels are inspected.

  o Sterilization of fermentation media: the media may be sterilized with steam injection in fermenters or media tanks.

• Hygienic processing:
  o Aseptical transfer from the lab stage and between fermentation steps.
  o Use of sterile air for aeration of the fermenters.

During the downstream processing hygienic conditions are also ensured by careful cleaning of equipment and hygienic controls at each step of the process. A polish filtration is performed as additional safety measure to keep level of microorganisms in the food enzyme preparation within specifications.

All the production steps are achieved following procedures executed by staff trained according to documented procedures complying with the requirements of the quality system

ii) In-Process Controls

In addition to these measures, in-process testing and monitoring is performed to guarantee a safe and optimal enzyme production process and a high quality product. The whole process is computer controlled, which reduces the probability of human errors in critical process steps.

These in-process controls include, but may not be limited to:
• Microbial controls: Absence of significant microbial contamination is analyzed by microscopy or plate counts before inoculation of both the seed and main fermentation, at regular intervals, and at critical process steps during fermentation and recovery.
• Monitoring of fermentation parameters (pH, temperature, feeding, aeration conditions,…). The values of these parameters are constantly monitored during the fermentation process. Deviations from the pre-defined values lead to investigations and adjustment, ensuring an optimal and consistent process.
• Monitoring of operational parameters during recovery steps (pH, temperature, enzymatic activity,…) throughout the entire downstream processing.

2.3.7 Stability of the enzyme during storage and prior to use

Food enzymes are formulated into various enzyme preparations in order to obtain standardized and stable products. Therefore, the stability depends on the type of formulation, not on the food enzyme as such.
The date of minimum durability or use-by-date is specified on the label of the food enzyme preparation. If necessary, special conditions of storage and/or use will also be stated on the label.

2.4 Product Composition and Specifications

2.4.1 Typical Quantitative Composition

The lipase enzyme preparation is generally produced in a solid form. The enzyme preparation does not contain any major food allergens from the fermentation media. Table 1 provides typical compositions as well as compositional analysis for 3 pilot batches (fermentation scale of 5 m^3).

Component	Typical composition	Batch results		
		PP 010	PP 012	PP 014
Lipase activity (LBLU[1]/g)	≥ 5,000 LBLU/g	10,795	6,008	9,289
Maltodextrin (%)	70-80%	75	75	71
Water (%)	≤ 6 %	4.1	3.8	4.6
Ash (%)	4-13 %	7.5	8.0	12.1
TOS [2] (%)	10-20 %	13.4	13.2	12.3

Table 1: Typical composition and compositional analysis of the enzyme solid preparation

[1] LBLU: Lallemand Baking Lipase Unit

[2] Total Organic Solids, defined as: 100% - water – ash – diluents

2.4.2 Specifications

Table 2 includes product specifications and analytical data of the four production batches, demonstrating compliance with the specifications.

Parameter	Specification	PP 010	PP 012	PP 014
Total aerobic plate count (CFU/g)	≤ 50,000	20,000	5,400	3,100
Coliforms (CFU/g)	≤ 30	< 10	< 10	< 10
E. coli (in 25g)	Not detected	Not Detected	Not Detected	Not Detected
Salmonella (in 25g)	Not detected	Not Detected	Not Detected	Not Detected
Production organism (in 1g)	Not detected	Not Detected	Not Detected	Not Detected
Antimicrobial activity	Not detected	Not Detected	Not Detected	Not Detected
Lead (mg/kg)	≤ 5	< 0.05	< 0.05	< 0.05

Table 2: Specifications and analytical data for 3 enzyme batches

2.5 Application and Use Levels

2.5.1 Technological Function

Lipase catalyzes the hydrolysis of triglycerides ester bonds into diglycerides and subsequently into monoglycerides and glycerol, as well as free fatty acids. It can be used in the manufacturing of baked goods such as bread, biscuits, buns and rolls, cakes, pancakes, wafers and waffles.

In baking processes, the lipase is added to the raw materials during the preparation of the dough. It is used to facilitate the handling of the dough, improve the dough structure and behaviour, as well as to reduce batter viscosity, thus contributing to an improved and consistent baking process. Lipase performs its technological function during dough or batter handling in order to contribute to an improved and consistent baking process and is then denatured by heat during the baking step.

2.5.2 Use Levels

The lipase enzyme should be used in baking at levels to achieve the desired technical effect and according to current good manufacturing practices (cGMP).
The amount of enzyme activity added to the raw material by the individual food manufacturer has to be determined case by case, based on the desired effect and process conditions. Therefore, the enzyme manufacturer can only issue a recommended enzyme use level, as a starting point for the individual food producer to fine-tune his process and determine the amount of enzyme that will provide the desired effect and nothing more. Consequently, from a technological point of view, there are no 'normal or maximal use levels' and lipase is used according to the *Quantum satis* principle.

The recommended use level depends on the application. Table 3 provides use levels commonly applied by the baking industry in the manufacturing of baked goods, as well as the corresponding TOS dose.

Type of food	Typical dose of lipase in flour (LBLU/kg flour)	Typical dose of lipase in flour (mg TOS/kg flour)*
Total white bread	250	3.7
Total whole grain and wheat bread	250	3.7
Buns and Rolls	250	3.7
Cake	2000	29.8

Table 3: Typical use levels of lipase in baked goods (non-exhaustive list)

* Dose in mg TOS/kg flour calculated based on average enzymatic activity (8,697 LBLU/g) and average TOS content (13.0 %) for the 3 batches

2.5.3 Enzyme Residues in the Final Food

The potential exposure of humans to the lipase enzyme is limited by the baked foods production process itself, whereby baking denatures the enzyme. In addition, enzymatic activity will be halted by the depletion of the substrate during the process. The enzyme does not exert any technological function in the final product.

3. Dietary Exposure

The Budget Method was used to obtain an estimate of the potential dietary exposure to the lipase enzyme intended for consumption for the general population on the basis that the enzyme processing aid is used in bread and other baking products.

The Budget Method is used as a screening tool and provides an overestimate of dietary exposure by using conservative assumptions in terms of use level and food consumption (FAO/WHO, 2009). This approach assumes that there is a maximum physiological amount of foods which can be consumed daily. Beverages were not included in the Budget Method calculation since the proposed uses of the lipase enzyme preparation is specific to food. The result is an estimate of the dietary exposure to the food enzyme preparation in the form of a Theoretical Maximum Daily Intake (TMDI). The assumptions of the Budget Method are outlined below.

Level of Consumption of Solid Foods

The FAO/WHO report on the Principles and Methods for the Risk Assessment of Chemicals in Food (FAO/WHO, 2009) specifies the standard values for food intakes at 0.05 kg/kg body weight/day (based on an estimated energy density of 2 kcal/g) for solid foods. Using the default body weight for adults of 70 kg, this is equivalent to an intake of 3.5 kg.

Level of Presence of Food Enzyme in Solid Foods

The amount of the lipase food enzyme preparation assumed to be present in solid foods is based on the maximum level of the food enzyme in flour (i.e. 29.8 mg TOS/kg flour). This conservative approach is made assuming that bread and other baking products prepared with the flour containing the food enzyme are only composed of flour.

Proportion of Solid Foods That May Contain the Food Enzyme

According to the budget method, a standard proportion of all solid foods of 12.5% are assumed to contain the food enzyme (FAO/WHO, 2009). As a conservative approach, 25% of solid foods may be made with the food enzyme (assumption for additives used in a wide range of foods (FAO/WHO, 2009)[2]). This assumes that a typical adult weighing 70 kg consumes 0.88 kg of solid food which are produced using the food enzyme preparation.

Theoretical Maximum Daily Intake of Enzyme

Based on conservative estimates of exposure calculated using the budget method, the TMDI of the lipase enzyme processing aid was calculated to be 0.373 mg TOS/kg body weight/day. The calculations for the derivation of the TMDI of the food enzyme preparation from all solid foods and the resulting total estimated intakes are presented in Table 4 below.

[2] Based on the assumptions of the FAO/WHO report on the Principles and Methods for the Risk Assessment of Chemicals in Food (FAO/WHO, 2009), 12.5% of solid foods are assumed to contain the ingredient produced using the food enzyme preparation, however this should be increased to 25% in the case of ingredients (produced using the food enzyme) used in a wide range of food categories.

Products	Level of Consumption of Solid Foods (kg/kg bw/day)	Proportion of Solid Foods Containing Food Enzyme (%)	Maximum Level of Food Enzyme in Solid Foods (mg TOS/kg)	Total Exposure to Food Enzyme [a] (mg TOS/kg bw/day)
Solid Foods	0.05	25	29.8	**0.373**

Table 4: TMDI of Lipase Based on the Maximum Use Levels in Solid Foods Using the Budget Method

bw = body weight; TMDI = Theoretical Maximum Daily Intake; TOS = Total Organic Solids

[a] Calculation: (Level of Consumption of Solid Foods) * (Proportion of Solid Foods Containing Food Enzyme/100) * (Maximum Level of Food Enzyme in Solid Foods)

Dietary Exposure to Any Other Substance Formed in or on Food

Lipase enzyme catalyzes the hydrolysis of triglycerides ester bonds into diglycerides and subsequently into monoglycerides and glycerol, as well as free fatty acids. These products are regular components of food and not expected to have any adverse effects on humans.

Dietary Exposure to Contaminants or By-products

Fermentation parameters including pH, aeration, temperature, and off-gas production are monitored during the fermentation process and deviations from the pre-defined values lead to adjustment to ensure an optimal and consistent process. Therefore, no harmful contaminants or by-products are expected. Furthermore, routine batch analysis is conducted to ensure the product complies with established specifications and is free of contaminants.

Conclusion on Dietary Exposure Assessment

The estimated human exposure to the lipase enzyme processing aid was calculated using the Budget Method, reflecting the proposed uses of the enzyme as a processing aid to be used in baked goods. The assumptions have been conservative to ensure there is no under-estimation of intakes of the food enzyme preparation. The Budget Method uses standard values to calculate the TDMI based on conservative assumptions regarding dietary intake of solid foods. In the assessment, the enzyme was assumed to be present at the maximum usage level in all applications of food and is assumed to be present at these levels in the final food as consumed.

The TMDI calculated for the lipase food enzyme using the Budget Method was 0.373 mg TOS/kg body weight per day based on the maximum intended use levels of the enzyme in the intended food uses. Furthermore, the consumer exposure to other substance formed in food is not anticipated to be of toxicological concern and contaminants/by-products are routinely monitored in the manufacturing product to ensure food-grade specifications are met.

Two GRAS notices (GRN 75[3] on a lipase derived from *Aspergillus oryzae* and GRN 631[4] on a lipase produced from *Trichoderma reesei*, both carrying the *F. oxysporum* lipase gene) reported the results of 13-week oral toxicity studies in rodents. The sequence of enzyme in these GRAS notices has 100% identity to the lipase enzyme produced by *Saccharomyces cerevisiae* LALL-LI.
In GRN 75, no observed adverse effect was reported at the highest dose of 830 mg TOS/kg body weight (bw)/day.
In GRN 631, a no observed adverse effect was reported at the highest dose of 1,000 mg powder/kg body weight (bw)/day.

Based on the lowest no observed adverse effect level (NOAEL) of 830 mg TOS/kg bw/day, the margin of safety is:

830 mg TOS/kg bw/day NOAEL ÷ 0.373 mg TOS/kg bw/day intake = 2,225

It should be stressed again that the TMDI used to calculate the margin of safety is based on very conservative assumptions and represents a highly exaggerated value. Overall, the human exposure to the lipase will be negligible. The enzyme is used as a processing aid and in very low dosages. Therefore, the safety margin calculation derived from this method is highly underestimated.

4. Self-Limiting Levels of Use

There are no proposed restrictions for the use of the lipase enzyme because the enzyme should be used in accordance with good manufacturing practices. See Section 2.5 for use levels.

5. Experience Based on Common Use in Food Before 1958

This part is not applicable to the notified substance.

6. Narrative

This safety assessment of the lipase from LALL-LI used in baking includes an evaluation of the safety of the production organism, the host organism, the donor and the enzyme. Each of these topics is addressed below.

[3] https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=75
[4] https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=631

6.1 Safety of the Production Organism *Saccharomyces cerevisiae*

The safety of the production organism is a prime consideration in assessing the probable degree of safety of an enzyme used in food (Pariza & Foster, 1983; Pariza & Johnson, 2001). The host strain used for modified *Saccharomyces cerevisiae* strain producing the lipase is a non-modified baker's yeast. This *Saccharomyces cerevisiae* strain was selected because of its use as a commercial strain in baker's yeast production and similarity to other baking 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; Muñoz *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 of *S. cerevisiae*

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).

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 928: Dried *saccharomyces cerevisiae* yeast fermentate for use as an ingredient in different foods
- GRN 842: Maltogenic α-amylase from *Geobacillus stearothermophilus* produced by *Saccharomyces cerevisiae* for use in baking
- GRN 841: *Saccharomyces cerevisiae* expressing L-lactate dehydrogenase from *Rhizopus oryzae* - for use in the fermentation of beer
- GRN 798: *Saccharomyces cerevisiae* strain yBBS002 - for use as a starter culture for brewing beer
- GRN 744: Steviol Glycosides with a High Rebaudioside M Content Produced by Microbial Fermentation - for use as a general-purpose sweetener in food
- GRN 626: Steviol glycosides produced in *Saccharomyces cerevisiae* – for use 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 - to reduce acrylamide production in a variety of grain-based foods, vegetable-based food (potato), and coffee and coffee substitutes
- 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 (US DHHS, 2019– Appendix C-III).

As EPA recognized in its Final Risk Assessment of *Saccharomyces cerevisiae* (February 1997; U.S. EPA, 1997 - p. 9), "Many 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). *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, 2007and 2008). Recent safety reviews by EFSA continue to support the QPS status of *S. cerevisiae* (EFSA BIOHAZ Panel, 2020 and 2021).

In its scientific opinion on the update of the list of QPS (EFSA BIOHAZ Panel, 2020) EFSA makes mention of some new reports of *S. cerevisiae* appearing as an opportunistic pathogen, but states that this brings no further concern regarding its QPS status. Moreover, the previous QPS qualifications has been confirmed, i.e. the absence of resistance to antimycotics used for medical treatment of yeast infections in cases where viable cells are added to the food or feed chain for *S. cerevisiae* strains able to grow above 37°C. As demonstrated by the results from the 3 batches showing the absence of viable cells in the lipase enzyme, no viable *S. cerevisiae* cells are added to the food chain in relation with the manufacturing process of the food enzyme, in agreement with the QPS qualification.

In a recent opinion, the EFSA panel on Food Contact Materials, Enzymes and Processing Aids (EFSA CEP Panel, 2021) evaluated as safe a maltogenic α-amylase produced from a genetically modified strain of *S. cerevisiae*, and considered this modified strain as qualifying for QPS.

6.2.3 Food Standards Australia New Zealand

Saccharomyces cerevisiae is recognized as a safe source for β-fructofuranosidase, and a genetically modified strain of *S. cerevisiae* is recognized as a safe source for maltogenic α-amylase as a processing aid (Schedule 18)[5].

6.2.4 Health Canada

Saccharomyces spp. is listed as a source microorganism for the production of invertase and lactase, and *saccharomyces cerevisiae* M17906 is listed as a source microorganism for the production of maltogenic α-amylase [6].

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

[5] https://www.legislation.gov.au/Details/F2021C01092
[6] List of permitted food enzymes (last update Oct.25, 2021): https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/5-enzymes.html

against hepatitis B was produced intracellularly in recombinant *S. cerevisiae* (McAleer *et al.*, 1984; Çelik & Çalık, 2012).

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 (IBS). 179 adults with IBS 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 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.

González Pereyra *et al.* (2014) reported on the probiotic *Saccharomyces cerevisiae* RC016 and tested 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 *Saccharomyces 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.* (2010) reviewed preclinical and clinical studies supporting the use of heat-killed whole recombinant *Saccharomyces cerevisiae* cells as therapeutic vaccines to treat cancer and infectious diseases. Wansley *et al.* (2008) 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'.

6.2.7 Conclusions

As summarized above, modern biotechnology delivers 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, Lallemand concludes that there is reasonable certainty of no harm to humans using the *Saccharomyces cerevisiae* as a production strain.

6.3 Safety of the Donor *Fusarium oxysporum*

The *Fusarium* genus was first introduced in 1809 (Abdel-Azeem *et al*., 2019). It is a cosmopolitan genus of filamentous ascomycete fungi. As a typical soil-borne genus, *Fusarium* species, especially F. oxysporum, are widely distributed and generally abundant in all types of soil around the world (Abdel-Azeem *et al*., 2019; Backhouse *et al*., 2001). *Fusarium oxysporum* is considered ubiquitous and is responsible for wilts or root rot disease in a wide range of crops (Abdel-Azeem *et al*., 2019). Like other fungi, Fusarium species are also widely used for production of bioactive metabolites, such as antioxidants and exopolysaccharides that see applications in food, feed, cosmetic, medicine, and pharmaceutical industries (Li et al. 2014). Fusarium species, including F. oxysporum, have also been

utilized for enzyme production in various industries including food and fuel industries (Thadathil, 2014; Ali and Vidhale, 2013; Suresh et al., 2014; Deshmukh and Vidhale, 2015; Xiros et al., 2011).

Fusarium species are best known as plant pathogens (Abdel-Azeem *et al*., 2019). Many FOSC (Fusarium oxysporum species complex) strains can infect plant roots without apparent effect or can even protect plants from subsequent infection (Abdel-Azeem *et al*., 2019). Fusaria also produce a diverse array of toxic secondary metabolites (mycotoxins), such as trichothecenes and fumonisins, which can contaminate agricultural products, making them unsuitable for food or feed (Abdel-Azeem *et al*., 2019; Mirocha *et al*., 1989).
Fusarium oxysporum belongs to the section Elegans of the genus *Fusarium* within the class of imperfect fungi Hyphomycetes. In general, these fungi are not regarded as primary human pathogens. Fusarium infections are opportunistic and rare in human and animals (Abdel-Azeem *et al*., 2019; Al-Hatmi *et al*., 2016). Some FOSC isolates have been identified as human pathogens causing infections in neutropenic individuals (Abdel-Azeem *et al*., 2019). Various enzymes products have been produced for food application by Fusarium oxysporum species, or by other organisms carrying genes from Fusarium oxysporum.

FDA issued Agency letters with no objections for three GRAS notifications using *Fusarium oxysporum*: Lipase derived from *Aspergillus oryzae* carrying a gene encoding lipase from *Fusarium oxysporum* (GRN 75); Lipase enzyme preparation from *Aspergillus oryzae* carrying a gene constructed from a modified *Thermomyces lanuginosus* lipase gene and a portion of the *Fusarium oxysporum* lipase gene (GRN 103); and triacylglycerol lipase from *Fusarium oxysporum* produced in *Trichoderma reesei* (GRN 631).

As mentioned previously, the lipase enzyme gene from *Fusarium oxysporum* 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 DNA fragments used in the construction of the expression cassette are well characterized and do not contain any undefined or harmful fragments.

In conclusion, we were unable to identify any risk factors for using *Fusarium oxysporum* as a gene donor for the lipase enzyme.

6.4 Safety of the Lipase Enzyme

Lipases are safely used in many industrial applications, including baking processes, since many years (Gerits et *al*. 2014, Chandra *et al*. 2020).

6.4.1 Regulatory Approvals/Safety Evaluations

Extensive regulatory approvals or safety evaluations support the safety of the lipase enzyme, including FDA, JECFA, Food standards Australia New Zealand (FSANZ), Health Canada and European Food Safety Authority (EFSA):

6.4.1.1 GRAS

FDA had no questions on the following GRAS notices:

- Lipase derived from *Aspergillus oryzae* carrying a gene encoding lipase from *Thermomyces lanuginosus* (GRN 43)
- Lipase from *Penicillium camembertii* (GRN 68)
- Lipase derived from *Aspergillus oryzae* carrying a gene encoding lipase from *Fusarium oxysporum* (GRN 75)
- Lipase from *Candida rugosa* (GRN 81)
- Lipase enzyme preparation from *Aspergillus oryzae* carrying a gene constructed from a modified *Thermomyces lanuginosus* lipase gene and a portion of the *Fusarium oxysporum* lipase gene (GRN 103)
- Lipase enzyme preparation from *Aspergillus niger* (GRN 111)
- Lipase enzyme preparation from *Aspergillus oryzae* (GRN 113)
- Lipase preparation from *Aspergillus niger* expressing a gene encoding a lipase from *Candida antartica* (GRN 158)
- Lipase enzyme preparation from *Rhizopus oryzae* (GRN 216)
- Lipase enzyme preparation derived from *Hansenula polymorpha* expressing a gene encoding a lipase from *Fusarium heterosporum* (GRN 238)
- Lipase enzyme preparation from a genetically modified strain of *Aspergillus niger* (GRN 296)
- Triacylglycerol lipase from *Fusarium oxysporum* produced in *Trichoderma reesei* (GRN 631)
- Triacylglycerol lipase from *Rhizopus oryzae* produced in *Aspergillus niger* (GRN 783)
- Lipase from *Aspergillus tubingensis* produced in *Trichoderma reesei* (GRN 808)

6.4.1.2 Joint FAO/WHO Expert Committee on Food Additives (JECFA)

JECFA first positively evaluated lipase enzyme produced by Aspergillus oryzae in 1974, with Acceptable Daily Intake listed as not specified (JECFA, 1974).
Lipase is listed on the Food Additive Index of CODEX General Standard for Food Additives (GSFA) (INS: 1104)[7].

6.4.1.3 Food Standards Australia New Zealand (FSANZ)

Lipases from *Aspergillus niger*, *Aspergillus oryzae*, *Candida cylindracea*, *Candida rugosa*, *Mucor javanicus*, *Penicillium roquefortii*, *Rhizopus arrhizus*, *Rhizomucor miehei*, *Rhizopus niveus*, *Rhizopus oryzae*, *Aspergillus oryzae* containing the lipase gene from *F. oxysporum*, *A. oryzae* containing the lipase gene from *Humicola lanuginosa*, *A. oryzae* containing the lipase gene from *Rhizomucor* miehei, *Hansenula polymorpha* containing the lipase gene from *Fusarium heterosporum*, *Aspergillus niger* containing a modified lipase gene from *fusarium culmorum*, *Trichoderma reesei* containing the

[7] https://www.fao.org/gsfaonline/additives/details.html?id=359&lang=en

lipase gene from *F. oxysporum*, and *T. reesei* containing the lipase gene from *Aspergillus tubingensis* are permitted enzymes in Australia New Zealand Food Standards Code[8].

6.4.1.4 Health Canada

Lipases from *Aspergillus niger*, *Aspergillus oryzae*, *Rhizopus oryzae*, *Rhizomocur miehei*, *Rhizopus niveus*, *Candida cylindracea*, *Candida rugosa*, *Mucor circinelloides* f. *circinelloides* (previous name: *Mucor javanicus*), *Penicillium roquefortii*, *Penicillium camembertii*, *Hansenula polymorpha* and *Trichodema reesei* have been approved for food use in Canada[9].

6.4.1.5 European Food Safety Authority (EFSA)

In Europe, even if currently no positive list of permitted enzymes has been published yet, EFSA has evaluated the following enzymes and considered them as safe for intended food uses:

- Lipase from a genetically modified strain of *Aspergillus oryzae* (strain NZYM-FL): https://www.efsa.europa.eu/en/efsajournal/pub/3762
- Lipase from a genetically modified strain of *Aspergillus oryzae* (strain NZYM-LH): https://www.efsa.europa.eu/en/efsajournal/pub/3763
- Lipase from a genetically modified strain of *Aspergillus oryzae* (strain NZYM-AL): https://www.efsa.europa.eu/en/efsajournal/pub/3778
- Triacylglycerol lipase from *Trichoderma reesei* (strain RF10625): https://www.efsa.europa.eu/en/efsajournal/pub/5837
- Triacylglycerol lipase from the genetically modified *Ogataea polymorpha* strain DP-Jzk33: https://www.efsa.europa.eu/en/efsajournal/pub/6048
- Triacylglycerol lipase from *Aspergillus niger* (strain LFS): https://www.efsa.europa.eu/en/efsajournal/pub/5630
- Triacylglycerol lipase from the genetically modified *Aspergillus niger* strain NZYM-DB: https://www.efsa.europa.eu/en/efsajournal/pub/6366
- Triacylglycerol lipase from the genetically modified *Aspergillus luchuensis* strain FL100SC: https://www.efsa.europa.eu/en/efsajournal/pub/6561

6.4.2 Allergenicity & Toxigenic Potential

Enzymes are proteinaceous molecules, and like other proteins, they possess the potential to elicit allergenic responses. As reported by Pariza and Foster (1983), "*Allergies and primary irritations from enzymes used in food processing should be considered a low priority item of concern except in very unusual circumstances*".

[8] https://www.legislation.gov.au/Details/F2021C01092

[9] https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/5-enzymes.html

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 (AMFEP, 1998). 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 inactivated during food processing and denatured proteins have 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. 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 lipase with a history of safe use have not raised safety concerns for food allergies (Bindslev-Jensen *et al.*, 2006).

To confirm that the lipase enzyme does not contain amino acid sequences similar to known allergens that might produce an allergenic response, a sequence homology search was conducted according to the approach outlined by Codex Alimentarius (2009) and EFSA (EFSA GMO Panel, 2010) in order to confirm the lack of potential for allergenic cross-reactivity. This search was conducted using the AllergenOnline[10] database version 21 and FASTA36. The database contains a comprehensive list of putative allergenic proteins developed *via* a peer-reviewed process for the purpose of evaluating food safety.

In accordance with the guidelines endorsed by Codex Alimentarius Commission (2009) and EFSA (EFSA GMO Panel, 2010) for the safety evaluation of newly expressed proteins from genetically modified plants and microorganisms, the database was searched using a sliding window of 80-amino acids sequences derived from the full-length amino acid sequence. According to the approach adopted by the Codex Alimentarius Commission and EFSA, 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.

Using this sequence homology search strategy, the lipase protein sequence showed no matches to known allergens (search performed on September 2, 2021).

[10] AllergenOnline is an allergen protein database containing 2,233 peer-reviewed allergenic protein sequences (Version 21; released on February 14, 2021) that is curated by the Food Allergy Research and Resource Program (FARRP) of the University of Nebraska. The database is available at: http://www.allergenonline.org/

A second homology search using the full length FASTA36 alignment of the amino acid protein sequence with known allergens on the AllergenOnline database (using default settings, i.e. E value cutoff = 1 and maximum alignments of 20) also showed no matches.
Finally, a sequence homology search was also conducted using the exact 8-mer approach, which is considered to be highly conservative, and did not identify any matches.

A bioinformatics search for similarity of lipase to known toxins was also performed. A custom FASTA database of known toxins was created by searching the UniProtKB database (https://www.uniprot.org/) with the terms "keyword:toxin". This search was performed on January 24, 2021 and resulted in a list of 246,266 proteins from both the manually annotated and reviewed Swiss-Prot database (563,972 records) and the computationally annotated and unreviewed TrEMBL database (209,157,139 records). On June 11, 2021 the amino acid sequence of *fusarium* lipase was queried against the custom toxin database using the BLAST function in the software Geneious Prime (The BLAST search used the BLOSUM62 matrix, gap cost (open extend) of 11 and 1, and word size 3. There were no hits, indicating that the sequence of fusarium lipase is not similar to any toxin sequence in the database.

As indicated above, enzymes are unlikely to be food allergens and the lipase enzyme has a history of use in food with no indication of safety concerns. In addition, the enzyme is typically removed or denatured during the baking process. Therefore, it is concluded that the expressed lipase enzyme is unlikely to be a concern with regard to food allergy or toxigenicity.

6.4.3 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; OECD, 1992; FAO/WHO, 1996; Pariza & Johnson, 2001) demonstrates the safety of these genetically modified production strains. 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."

The decision tree analysis for the lipase produced from *S. cerevisiae* LALL-LI, 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 loci 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 use in food products, such as bread.

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

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

The literature reports that *Saccharomyces cerevisiae* can be 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 and Hennequin (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 *Saccharomyces cerevisiae* fungemia at Hospital General Universitario. As part of the report, the authors searched MEDLINE for reports of *Saccharomyces cerevisiae* fungemia since 1966. Their search returned only 57 additional reported cases.

Since *Saccharomyces cerevisiae* is commonly used in the biotechnology industry, Murphy and Kavanagh (1999) also examined its potential pathogenicity. They also concluded that *Saccharomyces 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 *S. cerevisiae* into the blood stream are enteral translocation following antibiotic induced yeast overgrowth or CVC hub/insertion site contamination (Enache-Angoulvant and Hennequin, 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*," (EFSA BIOHAZ Panel, 2008, p.27) and EFSA maintains its QPS (Qualified Presumption as Safe) status for *S. cerevisiae* (EFSA BIOHAZ Panel, 2020). EFSA provides additional clarification stating, "the consumption of *Saccharomyces boulardii* (synonym of *S. cerevisiae*) by patients with fragile health may be considered as the possible origin of the infection" (EFSA BIOHAZ Panel, 2020). Even with the infrequent cases of fungemia associated with *Saccharomyces 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 *Saccharomyces 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* (U.S. EPA, 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).

6.6 Conclusions for GRAS determination

The following conclusions are made for the lipase enzyme from *Fusarium oxysporum* produced in a modified *Saccharomyces cerevisiae* for use in baking applications at the minimum level:

- 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, used in the production of human pharmaceuticals and the subject of several previous GRAS Notifications.

- The modified *Saccharomyces cerevisiae* production strain is derived from a native *Saccharomyces cerevisiae* yeast that has a safe history of use in the baking industry. The production strain has been determined to be substantially equivalent to the host strain with respect to overall performance such as growth and fermentation rates during propagation.

- The lipase enzyme produced by a 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.

- 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.

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

- No viable amounts of lipase enzyme remain in the bread products after baking.

- Extensive regulatory approvals or safety evaluations support the safety of lipase enzymes, including FDA, JECFA, FSANZ, Health Canada and EFSA.

Based on this evaluation and a review of the scientific literature, it is concluded that lipase enzyme from *fusarium oxysporum* produced in *Saccharomyces cerevisiae,* meeting appropriate food grade specifications and manufactured according to cGMP for food is GRAS for use in bread products and exempt from the premarket approval requirements based on scientific procedures.

7. List of Supporting Data and Information

Appendix 1: Lipase Production Process Flow Chart
Appendix 2: Safety Decision Tree for Lipase Enzyme

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

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

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

FDA USE ONLY

GRN NUMBER 001047	DATE OF RECEIPT Dec 24, 2021
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

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

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

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

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

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

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

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position or Title
Celia Martin	Regulatory Affairs Director

Organization *(if applicable)*: Lallemand Inc.

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

Name of Contact Person	Position or Title

Organization *(if applicable)*:

Mailing Address *(number and street)*:

City	State or Province	Zip Code/Postal Code	Country

Telephone Number	Fax Number	E-Mail Address

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

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

Lipase from Fusarium oxysporum produced by Saccharomyces cerevisiae

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 631

☐ b) GRAS Affirmation Petition No. GRP

☐ c) Food Additive Petition No. FAP

☐ d) Food Master File No. FMF

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

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

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

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? (see 21 CFR 170.225(c)(8) and 170.250(d) and (e))

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

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

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

☐ No

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

☐ Yes, a redacted copy of the complete submission

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

☐ No

SECTION D – INTENDED USE

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

The lipase enzyme is used as a processing aid during manufacturing of baked goods (to improve the dough structure and behaviour). It should be used at levels to achieve the desired technical effect and according to current good manufacturing practices (cGMP). The amount of enzyme used will vary with the food manufacturer and will be optimized for their process. No special subpopulation is expected to consume the enzyme.

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 Lallemand Inc.
(name of notifier)

has concluded that the intended use*(s)* of Lipase from Fusarium oxysporum produced by Saccharomyces cerevisiae
(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. Lallemand Inc.
(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.

1620 Prefontaine, Montreal, QC, H1W , Canada
(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)
Celia Martin — Digitally signed by Celia Martin Date: 2021.12.23 11:33:21 +01'00'	Celia Martin, Regulatory Affairs Director	12/23/2021

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)
	2021 (12-21) GRAS Notice Lipase produced by S.cerevisiae	Submission
	2021 (12-21) APPENDIX 1 - Lipase Production Process Flow Chart	Submission
	2021 (12-21) APPENDIX 2 - Safety Decision Tree for Lipase Enzyme	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.