GRAS Conclusion

A lipase from *Fusarium oxysporum* produced by *komagataella phaffii*

is Generally Recognized As Safe

for Use in Baking

September 4, 2023

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1. Signed Statements and Certifications

1.1 §170.225(c)(1) - Submission of GRAS notice

Lallemand Inc. is hereby submitting a GRAS (Generally Recognized as Safe) notice in accordance with subpart E of part 170 of the Code of Federal Regulation.

1.2 §170.225(c)(2) - The name and address of the notifier

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

1.3 §170.225(c)(3) – Appropriately descriptive term

Lipase food enzyme, produced from *Komagataella phaffii* expressing lipase from *Fusarium oxysporum*.

1.4 §170.225(c)(4) – 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 *Saccharomyces cerevisiae*, *Aspergillus oryzae* and *Trichoderma reesei.*

1.5 §170.225(c)(5) - Statutory basis for GRAS conclusion

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).

1.6 §170.225(c)(6) – Premarket approval

Lallemand Inc. has determined that its lipase enzyme produced by *Komagataella phaffii* 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.

1.7 §170.225(c)(7) – Availability of information

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

1.8 §170.225(c)(8) - FOIA (Freedom of Information Act)

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

1.9 §170.225(c)(9) – Information included in the GRAS notification

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

September 4, 2023

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 *Komagataella phaffii* strain expressing the gene encoding a lipase from *Fusarium oxysporum*.

International Union of Biochemistry (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: *Komagataella phaffii* LALL-LI2

Amino acid sequence: The total nucleotide and amino acid sequences have been determined. The lipase primary amino acid sequence is composed of 331 amino acids, corresponding to a predicted molecular weight of 35 kDa[1].

2.2 Identity of the Source

2.2.1 Host Microorganism

The host strain used for construction of the production strain LALL-LI2 is *Komagatella phaffi* ATCC 76273, also known as NRRL Y-11430 and CBS 7435. This is a methylotrophic yeast obtained from black oak (*Quercus kelloggii*), capable of using methanol as the sole carbon source.

This strain, formerly classified as *Pichia pastoris*, was reassigned as *Komagatella phaffii* following multigene sequence analyses (Kurtzman *et al*. 2009).

[1] The molecular weight was estimated using the Protein Molecular Weight calculator available at: https://www.bioinformatics.org/sms/prot_mw.html

Komagataella phaffii is widely used as a host for secretion of heterologous proteins, including enzymes, all the commonly used expression systems having the specific strain NRRL Y-11430 has an ancestor (Offei *et al*. 2022).

The history of the strain ATCC 76273 has been discussed in detail in GRN 737 (U.S. FDA, 2018).

2.2.2 Production Strain

The production organism LALL-LI2 is a strain of *Komagataella phaffii* 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 a 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 *Komagataella phaffii* by whole genome sequencing.

Taxonomic characteristics of the production strain:

Kingdom	Fungi
Phylum	*Ascomycota*
Class:	*Saccharomycetes*
Order:	*Saccharomycetales*
Family:	*Phaffomycetaceae*
Genus:	*Komagataella*
Species:	*Komagataella phaffi (*previously *Pichia pastoris)*

The production strain lineage is provided in Figure 1 below.

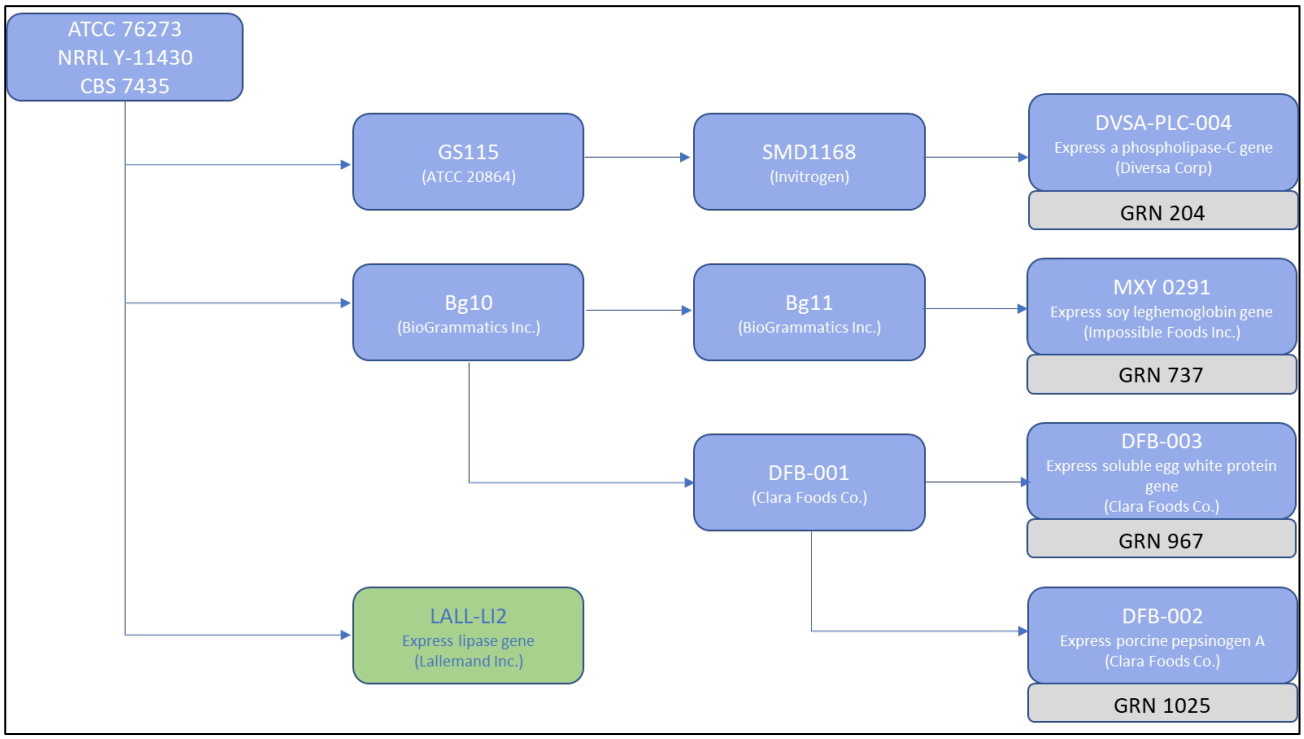

Figure 1: LALL-LI2 strain lineage

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 strain. Therefore, no DNA from the donor organism is present in the final strain.

Using the amino acid sequence of *F. oxysporum* lipase, excluding the first 15 amino acids (as the first 15 amino acids are predicted to be the secretory signal peptide, which would not be present in the mature protein[2]), a DNA sequence 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 new signal peptide was added to facilitate efficient secretion by *K. phaffii*. 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 purpose of the genetic modification is to enable the production strain *K. phaffii* LALL-LI2 to synthesise lipase from the donor *Fusarium oxysporum*.

[2]Signal peptide prediction was performed using SignalP-5.0 server https://services.healthtech.dtu.dk/services/SignalP-5.0/

The lipase DNA was stably integrated into the *K. phaffii* genome, under the regulation of native *K. phaffii* promoters and terminators, using classical genetic engineering approaches and molecular biology methods. No other heterologous DNA elements besides the described lipase are present in the LALL-LI2 strain genome.

PCR genotyping and whole genome sequencing (WGS) confirmed the correct insertion into the yeast genome at the intended location. WGS analysis also confirmed the absence of unintended foreign DNA sequence in the production strain as well as absence of antimicrobial resistance genes.

2.2.5 Stability of the Introduced DNA Sequences

The inserted DNA is integrated into the *K. phaffii* 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. Additionally, *K. phaffii* is a haploid organism whose capacity for homologous recombination is documented to be inefficient (Näätsaari et al., 2012). Therefore, the possibility of loss of the inserted DNA elements through chromosomal recombination is virtually absent in this host.

To determine the genotypic stability of the production strain *K. phaffii* LALL-LI2 throughout the propagation procedure, genomic DNA was isolated from the cells used for seeding the yeast propagation, and also from the final cream obtained at the end of the yeast propagation for 3 batches. 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 cassettes.

2.2.6 Antibiotic Resistance Genes

No antibiotic resistance genes were integrated in the production strain during strain engineering. Furthermore, absence of antibiotic resistance genes in the production strain was confirmed by whole genome sequencing.

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 enzyme from LALL-LI2. Therefore, the production organism does not end up in food.

2.3 Method of Manufacture

The lipase food enzyme is produced by fermentation of *Komagataella phaffi* LALL-LI2, followed by recovery (downstream processing), formulation and packaging. The manufacturing flow-chart is provided in Appendix 1.

2.3.1 Manufacturing in the Enzyme Production Plant

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).
The food enzyme is produced under a standard manufacturing process, in accordance with current Good Manufacturing Practices for food and the principles of hazard analysis and critical control points (HACCP), within certified manufacturing facilities with established procedures.

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 are ingredients that are accepted for general use in food, and classically used in the enzyme industry. 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 Food Chemicals Codex specifications except those raw materials which do not appear in the FCC. In this case, suitable ingredients are used, and internal specifications are established to meet the ones set forth by the FCC requirements.

2.3.3 Pure Culture Stage 1

Yeast propagation is initiated from frozen master stocks of pure culture maintained at -80°C in glycerol. The assurance that the production microorganism efficiently produces the desired enzyme

protein is key during the production process. Therefore, it is essential that the identity and purity of the production strain is controlled. Production of the required enzyme protein is based on a well-defined master cell bank and working stock culture. The cell line history and the production of a cell bank, propagation, preservation and storage is monitored and controlled following procedures. A stock culture is only accepted for production runs if its quality meets the required standards. This is determined by checking identity, viability, microbial purity and productivity of the culture.

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 small flask of sterile medium (autoclaved). This flask is cultivated in the laboratory to increase the numbers of growing cells prior to inoculating the culture into Pure Culture Stage 2.

2.3.4 Pure Culture Stage 2 and Fed-Batch

The yeast from the flask obtained from stage 1 is inoculated into a larger propagation vessel. The culture is then sequentially transferred into increasing fermenter volumes. The Pure Culture (PC) fermentations are conducted in batch mode, followed by one or more Fed Batch(s) (FB) fermentation, based on the amount of yeast cream needed.

During the fermentation steps the nutrients feeding rate, as well as the temperature and pH are controlled, according to the fermentation recipes, to provide the optimal growth with minimal ethanol production in the off-gas. The aeration rate (sterile air) during fermentation is controlled according to the fermentation recipes.
At the end of the FB fermentation (based on the recipe) the feeding is stopped to end the fermentation sequence.

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
2)Clarification, to remove most of the residual yeast cells
3) Concentration and purification, usually using ultrafiltration
4) Polish filtration - for removal of residual production strain organisms and as a general precaution against microbial contaminants.

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 Control.
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 purity criteria recommended for enzyme preparations as described in the Food Chemical Codex (FCC), 13th edition (USPC, 2022) and 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 are in place to guarantee microbiological hygiene and prevent contamination with microorganisms ubiquitously present in the environment (water, air, raw materials).

During the downstream processing hygienic conditions are also ensured by careful cleaning of equipment and hygienic controls at each step of the process. 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.

These in-process controls include, but may not be limited to:

- Microbial controls: Absence of significant microbial contamination is analysed 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, *etc.,*) 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, *etc.,*) throughout the entire downstream processing.

2.3.7 Stability of the enzyme during storage and prior to use

Food enzymes are formulated into various 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 scale-down pilot batches.

Ingredient	Typical composition	HH783P	B76Z6Y	BHC923
TOS* (%)	4 to 15	5.5	5.0	4.7
Maltodextrin (%)	80 to 90	87.1	87.0	86.3
Water (%)	2 to 6	5.6	5.3	5.1
TOTAL	100	100.0	100.0	100.0

*Total Organic Solids (TOS) define as: 100% - water – ash – diluents.

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

The final formulation components do not include or originate from sources that are major food allergens.

2.4.2 Specifications

Table 2 includes product specifications and analytical data for 3 independent scale-down pilot batches, demonstrating compliance with the specifications.

Parameter	Specification	Method of Analysis	Batch Nos.		
			HH783P	B76Z6Y	BHC923
Enzyme Activity					
Lipase (LBLU/g)	-	Internal Method	12,914	16,351	25,621
Lipase (LBLU/mg TOS)	-	Calculated	236.5	323.8	541.7
Microbiological Parameters					
Coliforms (CFU/g)	≤ 30	MFLP-64[3] or equivalent	< 10	< 10	< 10
Escherichia coli (/25g)	Absent	MFLP64 or equivalent	Absent	Absent	Absent
Salmonella (/25g)	Absent	NEOGEN® ANSR kit for Salmonella (AOAC certified)	Absent	Absent	Absent
Antimicrobial activity	Absent	Following JECFA guidelines[4]	Absent	Absent	Absent
Production Organism (/g)	Absent	Following EFSA guidelines[5]	Absent	Absent	Absent
Heavy Metals					
Lead (mg/kg)	≤ 5 [(a)]	AOAC 2011.14	0.028	0.047	0.025

LBLU = Lallemand Baking Lipase Unit; CFU = colony forming units

[(a)] The specification for lead is aligned with those in the most recent FCC enzyme monograph (FCC 13) and those established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006)[6].

Table 2: Specifications and analytical data for 3 enzyme batches

The methods used to establish the specifications have been validated for their intended purpose.

[3] https://www.canada.ca/en/health-canada/services/food-nutrition/research-programs-analytical-methods/analytical-methods/compendium-methods/laboratory-procedures-microbiological-analysis-foods-compendium-analytical-methods.html

[4] Joint FAO/WHO Expert Committee on Food Additive. COMBINED COMPENDIUM OF FOOD ADDITIVE SPECIFICATIONS, volume 4 (Analytical methods, test procedures and laboratory solutions used by and referenced in the food additive specifications), pp.122. http://www.fao.org/3/a0691e/a0691e.pdf

[5] EFSA CEP Panel. Scientific Guidance for the submission of dossiers on Food Enzymes, section 1.3.4.1 Viable cells of the production strain. *EFSA Journal* (2021), 19(10): 6851. https://doi.org/10.2903/j.efsa.2021.6851

[6] FAO/WHO, 2006. General specifications and considerations for enzyme preparations used in food processing in Compendium of food additive specifications. 67th meeting. FAO JECFA Monographs, 3, pp.63-67. http://www.fao.org/3/a-a0675e.pdf

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.

The benefits of the conversion of triglycerides with the help of lipase in baking can be summarized as follows:

- Facilitate the handling of the dough (improved extensibility and stability),
- Improve the dough structure and behavior during baking (consistent baking process),
- Ensure a uniform and slightly increased volume and an improved crumb structure of the bakery product, which might otherwise be impaired by industrial processing of the dough.

The lipase is added to the raw materials during the preparation of the dough, performs its technological function during dough handling, 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	1.01
Total whole grain and wheat bread	250	1.01
Buns and Rolls	250	1.01
Cake	2000	8.46

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

* Dose in mg TOS/kg flour calculated based on lowest enzymatic activity per TOS observed (236.5 LBLU/ mg TOS, lot HH783P; see section 2.4)

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. 8.46 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)[7]). 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 0.11 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.

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	5.6	**0.11**

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

[7] 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.

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 0.11 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, one on a lipase derived from *Aspergillus oryzae* (GRN 75, U.S. FDA 2001) and the other on a lipase produced from *Trichoderma reesei* (GRN 631, U.S. FDA 2016), 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 is similar to the lipase enzyme produced by *Komagataella phaffii* LALL-LI2.
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 can be calculated as:

830 mg TOS/kg bw/day NOAEL ÷ 0.11 mg TOS/kg bw/day intake = 7,545

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-LI2 used in baking includes an evaluation of the safety of the host organism, the donor, the production organism, the enzyme and the manufacturing process. Each of these topics is addressed below.

6.1 Safety of the Host Organism

The host strain used for construction of the production strain LALL-LI2 is *Komagatella phaffi* ATCC 76273, also known as NRRL Y-11430 and CBS 7435.
Komagatella phaffi NRRL Y-11430 as a long history of safe use as a production organism in the food, feed, and pharma industry (Offei *et al*. 2022).

Notably, phospholipase C enzyme (GRN 204, U.S. FDA 2006), soybean leghemoglobin (GRN 737; U.S. FDA, 2018), soluble egg-white protein (GRN 967, U.S. FDA 2021) and pepsin A (GRN 1025, U.S. FDA 2023a), are obtained from production organisms derived from this host (see strain lineage in section 2.2.2), and these 4 substances have received a no questions letter from the FDA.

Additionally, soy leghemoglobin preparation subject to GRN 737 has also been evaluated as safe by Health Canada for use as an ingredient in simulated meat products and other ground beef analogues. In its technical summary Health Canada acknowledged that the production organism was developed from a parental strain with an established history of safe use in the food industry[8].
It is also an authorised food produced using gene technology in Australia/New-Zealand as per schedule 26[9], for use in a meat analogue products. In its risk and technical assessment report (see application A1186[10]), FSANZ mentioned that *Komagataella p*haffii has been classified as a Biosafety Level 1 organism, has a recognised safe history of use for the production of food enzymes, and is neither pathogenic nor toxigenic. Furthermore, FSANZ could not identify any potential safety concerns associated with *K. phaffi* and no reports of adverse effects from products produced from *K. phaffi* strains were identified by a literature search.

Moreover, dried *Pichia pastoris* (now known as *Komagataella phaffii*) is also allowed for use in poultry food as per 21CFR573.750[11].

Finally, *Komagatella phaffi* has been included by EFSA in the list of organisms considered suitable for Qualified Presumption of Safety (QPS) approach for safety assessment with the qualification that it applies when the species is used for production purposes and no viable cells are found in the final

[8] https://www.canada.ca/en/health-canada/services/food-nutrition/genetically-modified-foods-other-novel-foods/approved-products/soy-leghemoglobin.html
[9] https://www.legislation.gov.au/Details/F2023C00138 [Last update Jan. 30, 2023]
[10] https://www.foodstandards.gov.au/code/applications/Pages/A1186.aspx
[11] https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=573.750

product (EFSA BIOHAZ Panel, 2023), which is the case for *K. phaffii* LALL-LI2, as demonstrated by the data provided in Table 2.

6.2 Safety of the Donor Organism

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 questions for four GRAS notifications using *Fusarium oxysporum*: Lipase derived from *Aspergillus oryzae* carrying a gene encoding lipase from *Fusarium oxysporum* (GRN 75, U.S. FDA 2001); 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, U.S. FDA 2002); Triacylglycerol lipase from *Fusarium oxysporum* produced in *Trichoderma reesei* (GRN 631, U.S. FDA 2016); And lipase enzyme preparation produced by *Saccharomyces cerevisiae* expressing a gene encoding a lipase from *Fusarium oxysporum* (GRN 1047, U.S. FDA 2023b), which is the same protein as the one subject to the current GRAS notice, expressed in a different microorganism.

More importantly, 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. Thus, the modified yeast contains only a limited introduced sequence pertaining to the gene of interest.

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

6.3 Safety of the Production Organism

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food (Pariza & Foster, 1983, Pariza & Johnson, 2001. If the organism is non-toxigenic and non-pathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, are safe to consume (IFBC, 1990). Pariza and Foster define a non-toxigenic organism as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a non-pathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances" (Pariza & Foster, 1983)

The genetic modification, corresponding to the integration of the *Fusarium oxysporum* lipase gene into the host strain genome, results in the production strain *K. phaffii* LALL-LI2, which differs from the host in its high lipase production capability.

WGS analysis demonstrated that LALL-LI2 consists wholly of *K. phaffii* sequence apart from the heterologous *F. oxysporum* lipase that was purposefully introduced, and no unintended foreign DNA sequence has been detected in the production strain.
Finally, whole-genome sequence data of the production strain confirmed the absence of antimicrobial resistance genes.

An evaluation of the modified *K. phaffii* LALL-LI2 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 this 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."

The decision tree analysis for the lipase produced from *K. phaffii* LALL-LI2, 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 location of the *K. phaffii* host 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.

6.4 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 lipase enzymes, 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)
  https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=43

- Lipase from *Penicillium camembertii* (GRN 68) https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=68
- Lipase derived from *Aspergillus oryzae* carrying a gene encoding lipase from *Fusarium oxysporum* (GRN 75, U.S. FDA, 2001)
- Lipase from *Candida rugosa* (GRN 81) https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=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; U.S. FDA 2002)
- Lipase enzyme preparation from *Aspergillus niger* (GRN 111) https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=111
- Lipase enzyme preparation from *Aspergillus oryzae* (GRN 113) https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=113
- Lipase preparation from *Aspergillus niger* expressing a gene encoding a lipase from *Candida antartica* (GRN 158) https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=158
- Lipase enzyme preparation from *Rhizopus oryzae* (GRN 216) https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=216
- Lipase enzyme preparation derived from *Hansenula polymorpha* expressing a gene encoding a lipase from *Fusarium heterosporum* (GRN 238) https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=238
- Lipase enzyme preparation from a genetically modified strain of *Aspergillus niger* (GRN 296) https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=296
- Triacylglycerol lipase from *Fusarium oxysporum* produced in *Trichoderma reesei* (GRN 631; U.S. FDA, 2016)
- Triacylglycerol lipase from *Rhizopus oryzae* produced in *Aspergillus niger* (GRN 783) https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=783
- Lipase from *Aspergillus tubingensis* produced in *Trichoderma reesei* (GRN 808) https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=808
- Lipase from *Penicillium camemberti* (GRN 908). https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=908
- Lipase enzyme preparation produced by *Saccharomyces cerevisiae* expressing a gene encoding a lipase from *Fusarium oxysporum* (GRN 1067; U.S. FDA, 2023b)

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)[12].

6.4.1.3 Food Standards Australia New Zealand (FSANZ)

Lipases from *Aspergillus niger*, *Aspergillus oryzae*, *Candida cylindracea*, *Candida rugosa*, *Mucor javanicus*, *Penicillium camembertii*, *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 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[13].

6.4.1.4 Health Canada

Various 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[14].

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 *Aspergillus niger* (strain LFS): https://www.efsa.europa.eu/en/efsajournal/pub/5630
- Triacylglycerol lipase from the genetically modified *Ogataea polymorpha* strain DP-Jzk33: https://www.efsa.europa.eu/en/efsajournal/pub/6048

[12] https://www.fao.org/gsfaonline/additives/details.html?id=359&lang=en
[13] https://www.legislation.gov.au/Details/F2023C00754 [Last updated July 9, 2023]
[14] https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/5-enzymes.html [Last updated Aug.3, 2023]

- 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
- Triacylglycerol lipase from the non-genetically modified Mucor circinelloides strain AE-LMH: https://www.efsa.europa.eu/en/efsajournal/pub/7755
- Triacylglycerol lipase from the non-genetically modified *Aspergillus luchuensis* strain AE-L: https://www.efsa.europa.eu/en/efsajournal/pub/7754
- Triacylglycerol lipase from the genetically modified *Saccharomyces cerevisiae* strain LALL-LI: https://www.efsa.europa.eu/en/efsajournal/pub/8091
  This lipase corresponds to the enzyme protein subject to the current GRAS notice, expressed in a different microorganism.
- Triacylglycerol lipase from the non-genetically modified *Rhizopus arrhizus* strain AE-TL(B): https://www.efsa.europa.eu/en/efsajournal/pub/8099

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*".

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 from LALL-LI2 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 in the EFSA scientific guidance for the submission of dossiers on food enzymes (EFSA CEP Panel, 2021) in order to confirm the lack of potential for allergenic cross-reactivity. This search was conducted using the AllergenOnline[15] database version 22 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.
The database was searched on July 28, 2023 using a sliding window of 80-amino acids sequences derived from the full-length amino acid sequence. According to the approach adopted in the EFSA guidance, 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.
Using this sequence homology search strategy, the lipase protein sequence showed no matches to known allergens.

A bioinformatics search for similarity of lipase to known toxins was 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 May 17, 2023 (utilizing UniProtKB release 2023_02 of May 3, 2023) and resulted in a list of 103,636 proteins from both the manually annotated and reviewed Swiss-Prot database (569,516 records) and the computationally annotated and unreviewed TrEMBL database (249,308,459 records). On the same day, the 331 amino acid sequence of the lipase from LALL-LI2 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 with at least 80% amino acid similarity and 70% coverage of the query sequence, which is the threshold recommended by EFSA in its statement on the requirements for whole genome sequence analysis of microorganisms intentionally used in the food chain (EFSA, 2021; section 3.5.3 - Identification of genes of concern). These results indicate that the sequence of lipase from LALL-LI2 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 and is obtained from a safe production strain. 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 Literature Search

Enzyme proteins do not generally raise safety concerns (Pariza & Johnson, 2001; Paris & Foster, 1983) (22). Pariza & Foster (1983) note that very few toxic agents have enzymatic properties.

[15] AllergenOnline is an allergen protein database containing 2,233 peer-reviewed allergenic protein sequences (Version 22; released on May 25, 2023) 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/

Papers identified through extensive literature searches on the safety of enzymes from microbial sources support the general assumption that industrial enzyme preparations from non-pathogenic organisms are safe and food enzyme preparations are considered unlikely to cause any acute toxicity, genotoxicity or repeated-dose oral toxicity (JECFA, 2020).

Additionally, A literature search on lipase was performed for the period from December 22, 2021 to August 25, 2023 (the period before was considered to be covered by the most recent GRAS notice on lipase, dated Dec. 21 2021; GRN 1067, U.S. FDA 2023b).
The search was conducted with Pubmed database and key words “lipase enzyme safety”, “lipase enzyme toxins”, and “lipase enzyme toxicity”. A total of 254 hits were found. The totality of the available abstracts was reviewed, and none was found to be inconsistent with our conclusion of the general recognition of safety of lipase enzyme. Based on the information above, it is concluded that lipase enzymes have a history of safe use in food and do not have toxic properties.

6.4.3 Safety Studies

Although food enzyme preparations are considered unlikely to cause any acute toxicity, genotoxicity or repeated-dose oral toxicity, the fermentation products of microorganisms remaining from the manufacturing process are of interest due to the potential presence of secondary metabolites that may induce toxicity when ingested (JECFA, 2020).

Nevertheless, based on the safety of the host, *K. phaffii* being non-pathogenic and non-toxigenic, and the fact that no concerns are raised by the genetic modification, and in addition no safety issues are raised by the manufacturing process, no specific safety studies have been conducted with the lipase from *K. phaffii* LALL-LI2.

Toxicity studies of food products obtained from production strains derived from the same host as LALL-LI2 have been described in GRN 204 (U.S. FDA 2006) and GRN 737 (U.S. FDA 2018). No safety concerns have been reported, and both have received a no questions letter from the FDA.

6.5 Safety of the Manufacturing Process

The lipase food enzyme is manufactured in accordance with current good manufacturing practices for food (cGMP) and the principles of hazard analysis and critical control points (HACCP), within certified manufacturing facilities with established procedures, using ingredients that are accepted for general use in foods, and under conditions that ensure a controlled fermentation.

The enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in the Food Chemical Codex (FCC), 13th edition (USPC, 2022). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (2006).

6.6 Conclusions for GRAS determination

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

- The modified *K. phaffi* production strain (LALL-LI2) is derived from a *K. phaffii* yeast that has a safe history of use in the food industry.

- LALL-LI2 is constructed via linear DNA transformation with synthetic gene 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.

- LALL-LI2 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. 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 enzyme preparation specifications.

- No viable amounts of lipase enzyme are expected to 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 *Komagataella phaffii* is GRAS for use in the manufacture of baked goods 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

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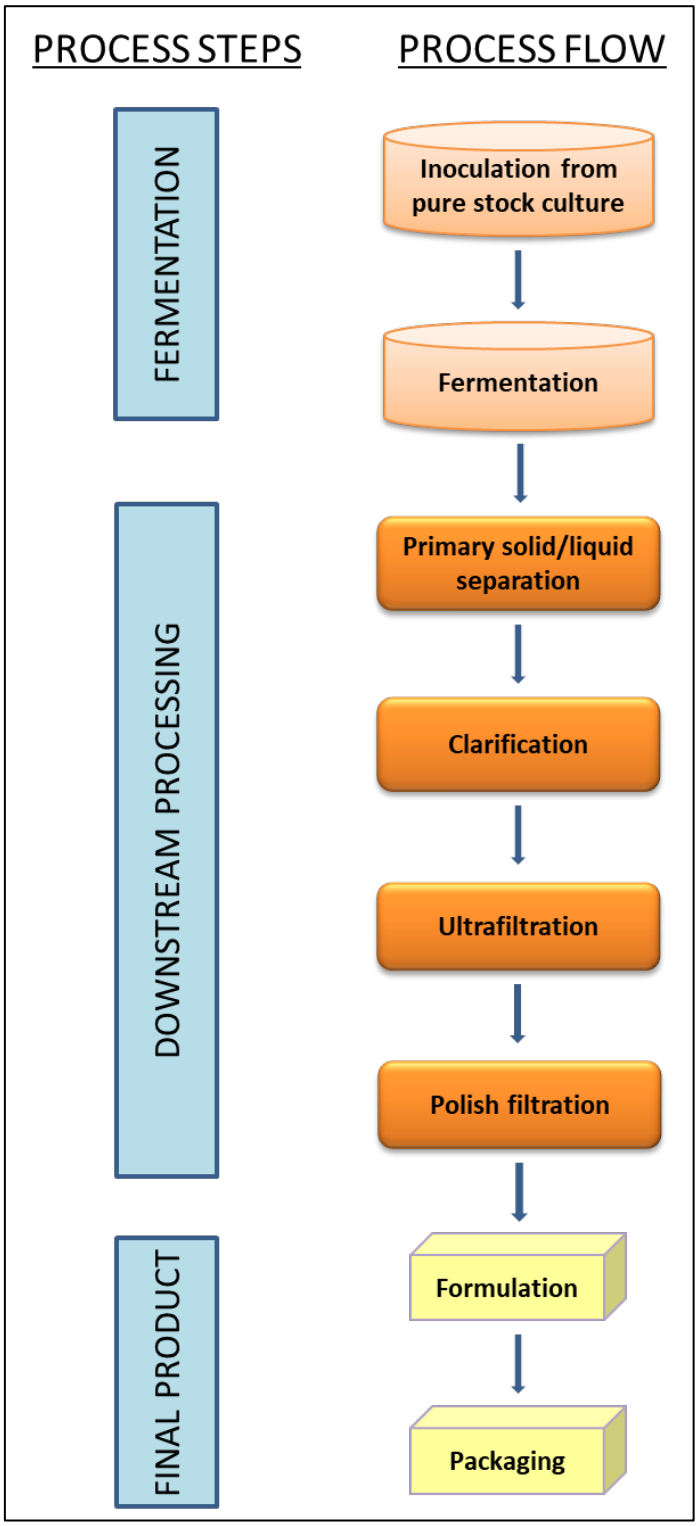
Appendix 1: Lipase Production Process Flow Chart



Appendix 2: Safety Decision Tree

1

- Is the production strain genetically modified? If yes, go to 2. ~~If no, go to 6.~~
- **Answer: Yes, the *K. phaffii* production strain is genetically modified.**

2

- Is the production strain modified using rDNA techniques? If yes, go to 3. ~~If no, go to 5.~~
- **Answer: Yes, the production strain was modified using recombinant DNA techniques, as described in Section 2.2.**

3a

- Do the expressed enzyme product(s), which are encoded by the introduced DNA, have a history of safe use in food? If yes, go to 3c. ~~If no, go to 3b.~~
- **Answer: The expressed enzyme product lipase from *Fusarium oxysporum*, has a history of safe use in food. In addition, the enzyme will be inactivated during baking.**

3c

- Is the test article free of tansferable antibiotic resistance gene DNA? If yes, go to 3e. ~~If no, go to 3d~~
- **Answer: Yes, the test article is free of antibiotic resistance genes as stated in section 2.2.6.**

3e

- Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products? If yes, go to 4. ~~If no, go to 12.~~
- **Answer: Yes, the introduced DNA is well characterized and free of attributes that would render it unsafe for constructing microorganisms used to produce food products.**

4

- Is the introduced DNA randomly integrated into the chromosome? ~~If yes, go to 5~~. If no, go to 6.
- **Answer: No.**

6

- Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure? If yes, the test article is ACCEPTED. ~~If no, go to 7.~~
- **Answer: Yes, the modified *komagataella phaffii* production strain is derived from a safe lineage as stated in section 2.2.2. Thus, it is concluded that the modified K. *phaffii* strain expressing the lipase enzyme is accepted under the decision tree guidelines as a safe strain lineage based on steps 1-6.**

Conclusion: ACCEPTED, under Decision Tree Guidelines

To Katie Overbey, Ph.D., M.S
Regulatory Review Scientist
Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
katie.overbey@fda.hhs.gov

April 17, 2024

Re: GRN1154_Questions_2024-04-03

Dear Dr. Overbey,
We thank the FDA for the review of our submission and are providing below reply to FDA's questions received via email on April 3, 2024, regarding GRN 1154.

1. For the administrative record please provide the correct CAS # for the lipase enzyme preparation.
The CAS number for the lipase enzyme preparation is 9001-62-1.

2. Page 6 contains the statement "all the commonly used expression systems having the specific strain NRRL Y-11430 has an ancestor." Please clarify what is meant by this statement.
This statement means that all the *K. phaffii* strains used as expression systems to produce heterologous proteins are derived from *K. phaffii* NRRL Y-11430, also known as *K. phaffii* ATCC76273 and *K. phaffii* CBS 7435. See for example the various quotes below (non exhaustive list), from the literature:
- Offei et al. 2022: "The yeast *Komagataella phaffii* (formerly called *Pichia pastoris*) is used widely as a host for secretion of heterologous proteins, but only a few isolates of this species exist and all the commonly used expression systems are derived from a single genetic background, CBS7435 (NRRL Y-11430)".
- Joseph et al. 2019: "All strains of *P. pastoris* are derived from the wild-type NRRL Y-11430".
- Gelissen et al. 2005: "All *P. pastoris* expression strains are derived from strain NRRL-Y 11430".

As one specific example, the soy leghemoglobin host strain *K. phaffii* Bg11 described in GRN 737 (US FDA 2018) is derived from strain NRRL Y-11430 (see strain lineage in figure 2 page 13).

3. Please state whether the production organism *Komagataella phaffii* LALL-LI2 has been deposited in a recognized culture collection.
The production strain *K. phaffii* LALL-LI2 has been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany) with deposit number DSM 34898.

4. Page 11 states that "All carriers are GRAS." We note that GRAS is specific to use; please confirm that all carriers used in your production process are GRAS for their intended use in this product.

We hereby confirm that all carriers used in the lipase production process are GRAS for their intended use in this product.

5. Please confirm that all formulation components of the final enzyme preparation are food-grade and authorized for their intended use by applicable US regulations.

We hereby confirm that all formulation components of the final enzyme preparation are food-grade and authorized for their intended use by applicable US regulations.

6. The manufacturing section lists several steps used in the recovery of the enzyme preparation, but the nature of these steps is not discussed. For the recovery steps listed on page 10, please indicate the general nature of each step (e.g. centrifugation, filtration, etc.).

Please find more details below regarding these steps:

1) Primary solid/liquid separation is usually performed by continuous centrifugation. Depending on the scale of the process and the site of operation, separation may also be conducted by filtration.

2)Clarification is generally achieved by cross flow microfiltration on tubular ceramic membranes. Alternatively, other clarification techniques may be used such as depth filtration by filter press or a cartridge filter can also be used, depending on the facility equipment.

3) Concentration and purification step is usually completed by ultrafiltration.

4) Polish filtration is accomplished using filters composed of cellulosic fibers, diatomaceous earth and resins which enables them remove contaminants based on both size exclusion and electrokinetic adsorption.

7. Please confirm if the dried form of the enzyme preparation is a powder.

We hereby confirm that the dried form of the enzyme preparation is a powder.

8. Please indicate the color of both the liquid and powder enzyme preparations.

The liquid enzyme preparation is brown, and the powder beige.

9. The batch analyses presented in Table 2 (page 13) show large variability in the determined lipase activity. Please provide a reasoning for this variation and provide a minimum specification for lipase enzyme activity.

For batches HH783P and B76Z6Y, the same downstream protocol was applied as for the lipase from *Saccharomyces cerevisiae* (GRN 1047, US FDA 2023). Nevertheless, as *K. Phaffii* fermentation medium contains a higher amount of minerals than the *S. cerevisiae* medium, the enzyme partly precipitated due to high ionic strength of the surrounding media, leading to some enzyme loss during the downstream processing.

The downstream processing has been adapted for batch BHC923 to obtain a lower ionic strength at key steps and therefore avoid enzyme precipitation, resulting in a higher lipase activity in the final product. Conductivity measurements are in place during the downstream processing to ensure appropriate ionic strength of the enzyme solution during the different steps.
In any case, the lipase activity in these 3 batches is higher than the minimum specification, set as 10,000 LBLU/g.

10. Page 13 lists AOAC method 2011.14 as the method used to measure lead. We note that AOAC method 2011.14 is validated for the analysis of nutrient elements (Ca, Cu, Fe, K, Mg, Mn, Na, P, and Zn) in infant formula and foodstuffs. Please provide results from an appropriate method for the analysis of lead in foods such as AOAC 2015.01 or FDA EAM 4.7.
The AOAC 2011.14 method is routinely used by Eurofins (internal method name ILCA-040/ILCA-069) to analyse lead content in various matrices. Please find along with this reply (provided as a stand-alone document) a statement from Eurofins (see 'Annex 1 - Eurofins statement on heavy metals analytical method'). As for methods AOAC 2015.01 or FDA EAM 4.7, the method used is based on digestion with nitric acid, followed by ICP-MS analysis.
Please let us know in case FDA would still consider the method AOAC 2011.14 as not appropriate to analyse lead in the lipase preparation. In case lead content needs to be re-analysed, would you consider the methods EPA3051a (digestion) and EPA6020b (ICP-MS analysis) as appropriate?

11. In line with FDA's "Closer to Zero" initiative, we request that specifications for lead reflect the amounts determined in the analysis of representative batches and be kept as low as possible.
We take note of this comment. The current specification for lead for the lipase from LALL-LI2 are aligned with those in the most recent FCC enzyme monograph (FCC 13) and those established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006).

12. The table on page 14 states that the maximum use level for the lipase is 8.46 mg TOS/kg flour and the table on page 16 states that the maximum use level is 5.6 mg TOS/kg. Please clarify the discrepancy.
The discrepancy comes from a typo in table 4, which has been updated as below.

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	8.46	**0.11**

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

13. On page 22 and 26, Lallemand cites GRN 1067 instead of GRN 1047. Please confirm if this was an error.
We hereby confirm that this is a typo and should be GRN 1047.

14. We note that, as GRAS notices are intended to cover the safety of the intended ingredient for consumption, the safety narrative should mainly focus on the safety of the actual enzyme preparation, with safe strain lineage serving as a corroborative component of safety. We note the following specific issues with the safety narrative:
a. Ultimately, the safety narrative contains very little data-based evidence on the safety of the notified enzyme preparation. Please provide a data-based narrative detailing the safety of the specific notified enzyme. If data on other lipases is used in this narrative, a discussion of the similarities between the notified enzyme and the enzymes being used to establish safety should be included. Further, evidence from the literature on safe human consumption should be cited and briefly discussed, if available.
The safety of the lipase protein from *fusarium oxysporum*, and therefore lipase protein from LALL-LI2, is discussed in reply to question 14 d below.

Additionally, we would like to emphasis here again that the safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food (Pariza & Foster, 1983, Pariza & Johnson, 2001. If the organism is non-toxigenic and non-pathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, are safe to consume (IFBC, 1990).

Komagataella phaffii has been included by EFSA in the list of organisms considered suitable for Qualified Presumption of Safety (QPS) approach for safety assessment with the qualification that it applies when the species is used for production purposes and no viable cells are found in the final product (EFSA BIOHAZ Panel, 2024). Based on an extensive literature search, EFSA BIOHAZ PANEL states that no safety concern has been reported for the *K. phaffii* species.

Additionally, a literature review was performed on March 25, 2024 regarding the safety of the host for LALL-LI2, i.e. *K. phaffii* ATCC 76273, aka NRRL Y-11430 and CBS 7435.

The 9 following searches were run using Google Scholar:

- pathogen pathogenicity "ATCC 76273". Returned eleven results.
- toxin toxigenicity toxicity "ATCC 76273". Returned two results.
- allergen allergenicity "ATCC 76273". Returned four results.
- pathogen pathogenicity "NRRL Y-11430". Returned forty-three results.
- toxin toxigenicity toxicity "NRRL Y-11430". Returned six results.
- allergen allergenicity "NRRL Y-11430". Returned twenty-five results.
- pathogen pathogenicity "CBS 7435". Returned twenty-nine results.
- toxin toxigenicity toxicity " CBS 7435". Returned two results.
- allergen allergenicity " CBS 7435". Returned three results.

A total of 125 references was retrieved using this strategy.
After removal of duplicates and non-peer reviewed references, a total of 47 references was obtained.
In various articles, *K. phaffii* NRRL Y-11430/ATCC 76273/CBS 7435 is described as the most commonly used *K. phaffii* strain for industrial applications and the ancestor of the lineage, other *K. phaffii* strains used industrially being commonly derived from it.
From this literature review, we have retrieved no information suggesting any risk regarding the potential strain pathogenicity and capacity to produce toxins and allergens. On the contrary, *K. phaffii* species is generally described as safe for the production of recombinant proteins, as not harboring pathogens, viral inclusions, or pyrogens.
Results from this literature search corroborate the safety of *K. phaffii* ATCC 76273 and therefore the one from LALL-LI2, as no concerns are raised by the genetic modification.

Moreover, as shown in figure 1 in GRN 1154, the production strain for soy leghemoglobin is obtained from a host strain (Bg11) which is also derived from ATCC 76273.
This relation is also acknowledged in the scientific literature, for example in Frazer et al. (2018): "The *P pastoris* LegH production strain MXY0291 was derived from the well-characterized parent strain NRRL Y-11430".
Therefore, both host strains for the lipase from LALL-LI2 and soy leghemoglobin from MXY0291 are from the same lineage.
Soy leghemoglobin has been the subject of a no questions letter from the FDA (GRN 737, US FDA 2018). Health Canada has also acknowledged the safety of the parental strain engineered to obtain the soy leghemoglobin production strain: "The *P. pastoris* production organism was developed from a parental strain with an established history of safe use in the food industry."[1]

The manufacturing process for both the lipase from *K. phaffii* LALL-LI2 and soy leghemoglobin is based on submerged fermentation followed by downstream processing to isolate and concentrate the desired product[1,Error! Bookmark not defined.].

Based on the explanation above, we are confident that the following toxicity data on soy leghemoglobin are acceptable for read-across to support the safety of the strain LALL-LI2, and therefore to support the safety of the lipase preparation from this strain:

Genotoxicity - Bacterial Reverse Mutation Assay
A bacterial reverse mutation test (Ames) was performed to assess the mutagenic potential of soy leghemoglobin and related preparation, according to OECD guideline 471 (Fraser *et al*., 2018).
Five bacterial strains were tested, in triplicate: *Salmonella typhimurium* (*S. typhimurium*) TA1535, TA1537, TA98, TA100, and *E. coli* WP2 uvrA. The main test was conducted using the plate incorporation method, both

[1] https://www.canada.ca/en/health-canada/services/food-nutrition/genetically-modified-foods-other-novel-foods/approved-products/soy-leghemoglobin/document.html

in the presence and absence of metabolic activation (S9), and the confirmation test was conducted using the pre-incubation method.
The maximum concentration tested was 74,000 µg/plate, corresponding to a maximum soy leghemoglobin concentration of 5,000 µg/plate.
For both the main test and the confirmation test, no signs of toxicity were reported in any of the 5 bacterial strains tested, in the presence or absence of S9.
Authors conclude that the soy leghemoglobin preparation is non-mutagenic at a soy leghemoglobin concentration up to 5,000 µg/plate.
Non mutagenicity of soy leghemoglobin preparation has been recently confirmed in a second bacterial reverse mutation assay (Reyes et al., 2023).

Genotoxicity - In Vitro Mammalian Chromosome Aberration Test in Human Lymphocytes
A chromosome aberration assay was achieved to evaluate the potential of soy leghemoglobin and related preparation to induce structural chromosome aberrations in human lymphocytes, according to OECD guideline 473 (Fraser *et al*., 2018).
The study was conducted using human peripheral blood lymphocytes (HPBL), obtained from healthy nonsmoking donors who had no recent history of exposure to genotoxic chemicals and radiation, in both the absence and the presence of the chemically induced rat liver S9 metabolic activation system. HPBL cells were exposed to the soy leghemoglobin preparation either for 4 hours in the presence or absence of S9 (experiment 1) or for 24 hours in the absence of S9 (experiment 2). For both experiments, the mitotic index was determined, and the proliferation index of selected samples (negative control and high doses of soy leghemoglobin) was calculated.

In experiment 1, the maximum concentration tested was 74,000 µg/mL of soy leghemoglobin preparation, corresponding to a maximum soy leghemoglobin concentration of 5,000 µg/mL.
Without S9 metabolic activation, the mitotic index decreased to below 70% of the negative control at high concentrations of soy leghemoglobin but remained above the 45% of control threshold that is recommended to accurately measure chromosome aberrations. No mitotic index decrease was observed in the presence of S9 metabolic activation.
Additionally, no significant difference in the proliferation index was observed under either condition (S9 activation or not) between the control and 5,000 µg/mL dose. Moreover, no significant increase in cells with structural or numerical chromosome aberrations was observed up to the highest dose of 5,000 µg/mL.

In experiment 2, precipitation of the product was observed for concentrations of ≥ 500 µg/mL, due to longer incubation time. Additionally, the mitotic index values versus the control decreased below the 45% threshold at concentrations > 1,000 µg/mL. Therefore, only concentrations up to 74,000 µg/mL of soy leghemoglobin preparation (1,000 µg/mL soy leghemoglobin) were evaluated for chromosome aberrations in experiment 2.

A proliferation index value of 79% and 72% relative to the control was observed for the does of 500 and 1,000 µg/mL, respectively, not related to chromosome aberrations. In fact, no significant increase in cells with structural or numerical chromosome aberrations was observed up to the highest dose of 1000 µg/mL.

Authors conclude that the soy leghemoglobin preparation is considered non clastogenic in the *in vitro* mammalian chromosome aberration test using HPBL.
Non clastogenicity of soy leghemoglobin preparation has been recently confirmed in a second *in vitro* mammalian micronucleus assay using HPBL (Reyes et al., 2023).

90-Day Oral Study in Rats (with a 28-Day Recovery Study)
Oral toxicity of the soy leghemoglobin preparation has been assessed in a 90-day oral study in Sprague–Dawley® rats, followed by a 28-day recovery period. The study was performed according to OECD guideline 408 (Reyes et al., 2023).
Four doses (including control) were evaluated, up to maximum targeted dose of 5,625 mg/kg bw/day of soy leghemoglobin. Ten animals per sex and per dose group were included. Five additional animals remained on the study for an additional 28-day recovery period for control and high dose group.
All animals were observed once a day for any sign of toxicity, survivability, and behavior and weekly for detailed clinical observations. Body weights were recorded twice during acclimation, including prior to test initiation on Day 0, and weekly thereafter until Day 91 (main test) and on Day 119 (recovery).

No mortalities occurred over the course of the study. All clinical observations noted were considered incidental and of no toxicological relevance and there were no clinical observations attributed to the soy leghemoglobin. Also, there were no changes in body weight.
A no observed adverse effect level (NOAEL) of 4,798.3 and 5,761.5 mg/kg/day, the maximum level tested for male and female rats, respectively has been established.
Authors conclude that under the conditions tested, the soy leghemoglobin preparation is not toxic.

b. The notifier mentions other GRAS notices similar to the notified enzyme preparation. If the notifier intends to incorporate safety data from previous GRNs submitted to the agency, we request that a summary of pivotal safety information from these prior notices be included, along with citations, since each GRN is intended to stand alone. Further, we request a discussion of the similarities between the notified enzyme and the referenced enzymes.
Summary of safety data for soy leghemoglobin (GRN 737, US FDA 2018), along with explanation on the relation between the production strain and the lipase production strain LALL-LI2, are discussed above in reply to question 14 a. These safety data sustain the safety of the production strain LALL-LI2.

Regarding the safety of the protein itself, the relation between the lipase from *K. phaffii* LALL-LI2 and the one from *Trichoderma reesei* RF10625 (GRN 631, US FDA 2016) and *Aspergillus oryzae* MStr110 (GRN 075, US FDA 2001) is discussed in reply to question 14 d below, and we consider that the protein from LALL-LI2 is closely related to the ones subject to GRN 075 and GRN 631, and therefore safety profile of the 3 proteins can be considered as equivalent.

The lipase subject to GRN 075 was evaluated in a bacterial reverse mutation test (Ames) to assess the mutagenic potential of the enzyme preparation, according to OECD guideline 471. Strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* (WP2 uvrA) were tested, both in the

presence and absence of metabolic activation (S9), at a maximum concentration of 5 mg per ml (*S. typhimurium*) or per plate (*E. coli*). It was concluded that the lipase enzyme preparation is non-mutagenic.
A chromosome aberration assay was also performed to evaluate the potential of the enzyme to induce structural chromosome aberrations in human lymphocytes, according to OECD guideline 473. Test was performed in both in the absence and presence of metabolic activation by a rat liver post-mitochondrial fraction (S-9), at a maximum dose of 5 mg/ml. It was concluded that the enzyme preparation did not induce chromosome aberrations in cultured human lymphocytes.
The enzyme was also tested in a 90-day oral toxicity study in rats (10 animals per sex), according to OECD guideline 408, at doses corresponding to 83, 249 and 830 mg Total Organic Solids (TOS)/kg bw/day. No toxicologically significant changes were observed, and the no-observed-adverse-effect level (NOAEL) was set as 830 mg TOS/kg bw/day.
These results demonstrate the safety of the enzyme preparation, including the safety of the protein itself.

The lipase subject to GRN 631 was evaluated in a bacterial reverse mutation test (Ames) to assess the mutagenic potential of the enzyme preparation, according to OECD guideline 471. Strains of *Salmonella typhimurium* (TA98, TA100, TA 102 TA1535 and TA1537) were tested, both in the presence and absence of metabolic activation (S9), at a maximum concentration of 5 mg per plate. It was concluded that the lipase enzyme preparation is non-mutagenic.
A chromosome aberration assay was also performed to evaluate the potential of the enzyme to induce structural chromosome aberrations in human lymphocytes, according to OECD guideline 473. Test was performed in both in the absence and presence of metabolic activation (S-9), at a maximum dose of 5.3 mg/ml (corresponding to 5.0 mg/ml TOS). It was concluded that the enzyme preparation did not induce chromosome aberrations in human lymphocytes.
The enzyme was also tested in a 13-week oral toxicity study in rats (10 animals per sex), according to OECD guideline 408, at doses corresponding to 50, 200 and 1,000 mg of enzyme preparation/kg bw/day. No toxicologically significant changes were observed, and the no-observed-adverse-effect level (NOAEL) for the enzyme preparation was set as 1,000 mg /kg bw/day.
These results demonstrate the safety of the enzyme preparation, including the safety of the protein itself.

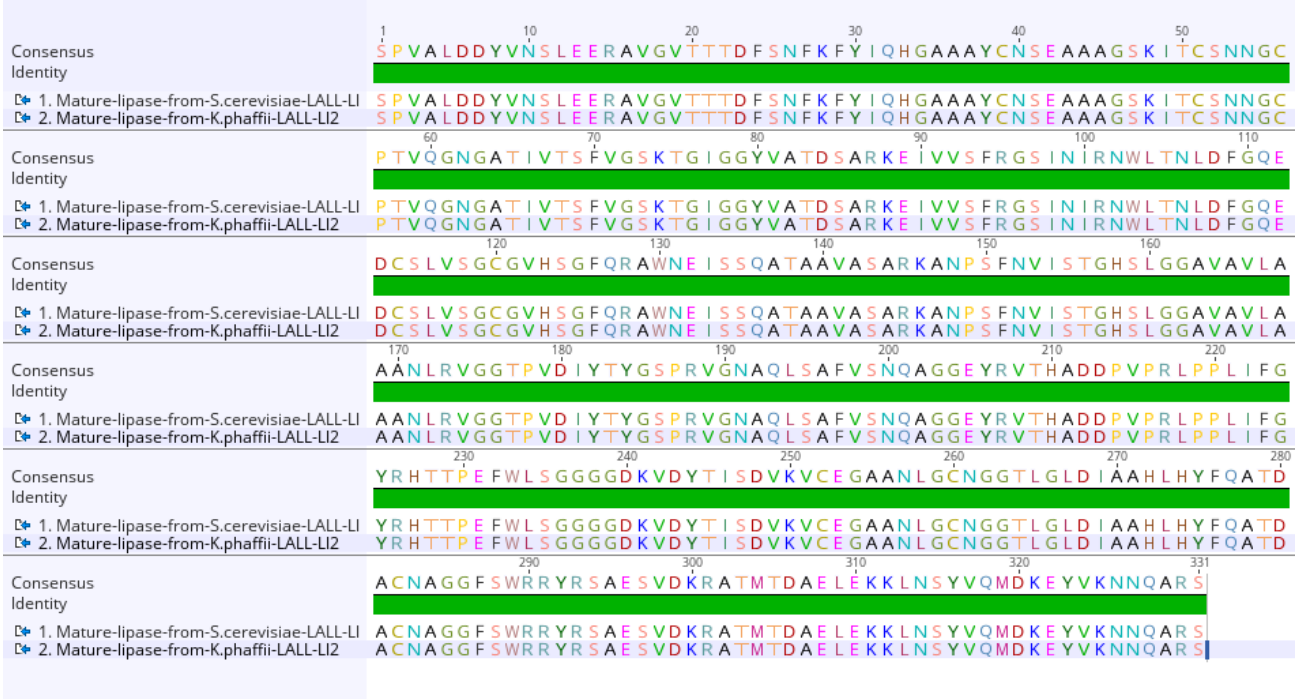
c. The notifier includes listings of other regulatory body approvals of lipase enzyme preparations with no additional discussion. We note that the approval of an ingredient by another regulatory body is not sufficient to establish safety. If this information is intended as a pivotal component of safety, a discussion of the similarity between the other approved enzymes, including identity and use, should be discussed.
For example, the lipase from *Saccharomyces cerevisiae* LALL-LI, which also corresponds to native *Fusarium oxysporum* lipase protein but produced from a different microorganism, has been positively evaluated or permitted in various areas and is therefore considered as safe, including:

- Canada, for use in bread, flour, whole wheat flour and unstandardized baking products[2].
- EU, for use in baking processes (EFSA CEP Panel, 2023)

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

- USA, in the manufacture of baked goods (GRN 1047, U.S. FDA 2023). A no questions letter was received from the FDA on May 12, 2023.

An amino acid sequence alignment between the previously approved lipase produced from *S. cerevisiae* LALL-LI and the notified lipase produced from *K. phaffii* LALL-LI2, show 100% alignment.



d. Lallemand mentions and discusses GRAS notifications for several lipases that previously received a No Questions Letter (GRN 75 and GRN 631). On pg. 17, Lallemand states "The sequence of enzyme in these GRAS notices [GRN 75 and 631] is similar to the lipase enzyme produced by Komagataella phaffii LALL-LI2." Further, Lallemand provides a margin of exposure (MOE) based on a no-observed-adverse-effect level (NOAEL) of 830 mg TOS/kg bw/d from a toxicological study using *F. oxysporum* lipase produced in *A. oryzae* (GRN 75) as test article and the exposure estimate calculated in this notice.

We note, however, that in order to validate a MOE derived from a study that did not specifically use the article of commerce as test article, notifiers typically provide sufficient detail and narrative to justify a read-across approach (e.g., bridging the similarities and differences between the article of commerce and the test article).
Please provide sufficient detail (other than the fact that lipases in GRN 75 and 631 are derived from F. oxysporum) to justify why Lallemand believes toxicological studies described in GRN 75 and/or 631 are appropriate to establish safety of the lipase described in GRN 1154. We note that information such as percent identity between the lipase described in GRN 1154 and lipases described in GRN 75 and 631 (or other lipases that have received a No Questions Letter) may be useful, along with a discussion on whether any differences in identity are expected to impact safety.

As discussed in reply to question 14c, it has been demonstrated that the lipase from *K. phaffii* LALL-LI2 is 100% similar to the one from *S. cerevisiae* LALL-LI.

Additionally, the predominant enzyme sequences present in the lipase from LALL-LI, RF10625 (GRN 631, US FDA 2016) and MStr110 (GRN 075, US FDA 2001) have been determined through mass spectrometry-based proteomic analysis (LC-MS/MS). Rehydrated samples were proteolyzed with trypsin according to standard procedures and the resulting peptide hits from LC-MS/MS were analyzed through Proteome Discoverer. Searches were performed against Uniprot proteomes.

Resulting alignments provided approximately 85% coverage and identified the lipase from *Fusarium oxysporum* as the dominant enzyme in each product. The region missing coverage is underlined on the lipase amino acid sequence below. Given that it is a larger-than-typical tryptic peptide (40 residues between the K and R tryptic cleavage sites), detection of this peptide is challenging by LC-MS/MS. Lack of coverage here means differences in this region cannot be excluded. However, given that it is the site of two cysteine disulfide bridges required for structural stability, substantial differences are unlikely as they may fail to preserve function. Coverage was obtained for all other regions of the sequence below and match the expected amino acid sequence of the *F. oxysporum* lipase. Based on the regions within coverage, no mutations or differences were identified between the lipase enzymes from the three sources. No additional peptides from other lipases were identified in the samples either. The inference from this analysis is that the lipase produced by *Trichoderma reesei* RF10625 and the lipase produced from *Aspergillus oryzae* MStr110 are the same as the lipase enzyme expressed in *S. cerevisiae* LALL-LI, and therefore also the same as the lipase from *K. phaffii* LALL-LI2.

SPVALDDYVNSLEERAVGVTTTDFSNFKFYIQHGAAAYCNSEAAAGSKITCSNNGCPTVQGNGATIVTSFVGSKTGIGGYVA
TDSARKEIVVSFRGSINIRNWLTNLDFGQEDCSLVSGCGVHSGFQRAWNEISSQATAAVASARKANPSFNVISTGHSLGGAV
AVLAAANLRVGGTPVDIYTYGSPRVGNAQLSAFVSNQAGGEYRVTHADDPVPRLPPLIFGYRHTTPEFWLSGGGGDKVDYT
ISDVK<u>VCEGAANLGCNGGTLGLDIAAHLHYFQATDACNAGGFSWR</u>RYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVK
NNQARS

Therefore, we are confident that toxicological studies described in GRN 75 and GRN 631 are appropriate to establish the safety of the lipase protein described in GRN 1154.

Should you require any further information or have any questions regarding GRN 1071, please do not hesitate to contact me.

Yours sincerely,

[redacted]

Celia Martin, PhD
Regulatory Affairs Director
Lallemand Inc.

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April 12th, 2024

Object: Metal analysis (ILCA-040/ILCA-069)

Application Domain

This procedure applies to the solution of food samples, natural health products (NHP) and cosmetics for ICP-MS analysis using a microwave oven.

Abstract

The decomposition of the different matrices is carried out by acid oxidation. For these purposes, nitric acid (HNO3 Trace Metal) is used. The samples are digested at a temperature above the boiling temperature of the acids. Reaching this temperature is possible because digestion takes place under pressure between 25 to 30 bar.

The assay is performed using an argon plasma ionizing source mass spectrometer (ICP-MS). The sample is introduced to an argon plasma via a peristaltic pump and nebulizer. The metals contained in the sample are atomized then ionized in the plasma. The produced ions are introduced into the mass spectrometer chamber where they are directed by a focusing system, separated by a quadrupole, to finally be captured by an electron multiplier (detector).

The concentration of an element at a specific mass is determined by comparison between the quantities of ions captured between the sample and standard solutions.

Reported limits

Parameters	RL	RL	Parameters	RL	RL
	Food	NHP & Cosmetics		Food	NHP & Cosmetics
	mg/Kg	mg/Kg		mg/Kg	mg/Kg
Ag	0.02	0.02	Mg	5.00	5.00
Al	1.00	2.00	Mn	0.10	0.20
As	0.02	0.03	Mo	0.05	0.10
B	0.50	1.00	Na	10.00	20.00
Ba	0.05	0.05	Ni	0.07	0.10
Be	0.25	0.50	P	10.00	10.00
Bi	0.02	0.05	Pb	0.02	0.03
Ca	20.00	20.00	Sb	0.01	0.05
Cd	0.01	0.01	Se	0.15	0.20
Co	0.01	0.01	Sn	0.05	0.10
Cr	0.03	0.05	Sr	0.20	0.50
Cu	0.20	0.20	Ti	0.10	0.50
Fe	5.00	5.00	Tl	0.01	0.05
Hg	0.01	0.01	U	0.01	0.10
K	10.00	10.00	V	0.10	0.10
Li	0.50	0.50	Zn	1.00	0.50

Sample quantity

2 g for solids
10 mL for liquids

Turn Around Times

5 business days for food matrices
10 business days for NHP and cosmetics

Reference method

AOAC (2011). AOAC Official Method 2011.14 6p *Minerals and Trace Elements in Fortified Food Product, Milk and Milk Products, Infant Formula, and Adult Nutritionals: Microwave Digestion and Inductively Coupled Plasma-Atomic Emission Spectrometry*

Note

The external reference method (AOAC 2011.14) is used as the basis of our internal methodology.

The external reference method provides Guidance for digestion and dosage for food, natural health product and cosmetic matrices.

The external reference method validation data is to indicate what is expected and achievable by the method. Some differences observed between validations (ref and lab) are expected due to environment, instruments used, different chemists and consumables used. In addition, certain parameters have been added following rigorous validation according to our acceptability criteria. The parameters are: Ag, Al, As, B, Ba, Be, Bi, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Sb, Se, Sn, Sr, Ti, Tl, U, V & Zn.

Each year we participate in various proficiency testing programs to confirm the effectiveness of our internal method. Food matrices containing unknown concentrations of the different parameters are analyzed, and the results are submitted to under the proficiency testing programs. We then receive a final report which attests to our level of performance.

If you have any questions, do not hesitate to contact us.

Best regards,

From: Martin Celia
To: Overbey, Katie
Subject: [EXTERNAL] RE: GRN 1154 - Additional Question
Date: Wednesday, June 19, 2024 2:49:29 AM
Attachments: image002.png

Hi Katie,

I am hereby providing the reply to your additional question, as response to your email, please see below:

1. In the amendment from April 17, 2024, Lallemand stated that their specification for lead for the lipase enzyme preparation is aligned with the Food Chemical Codex (13th edition, FCC 13) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA 2006). The results from three batch analyses for lead presented in GRN 1154 are two orders of magnitude lower compared to the FCC13 and JECFA specification of <5 mg/kg. Please re-consider reducing the specification for lead to reflect the batch analyses presented in Table 2.

Lallemand answer: We hereby acknowledge to adapt the specification for lead for the lipase subject to GRAS Notice 001154, as ≤ 1 ppm, to better reflect the results from batch analyses presented in the Notice.

I kindly ask you to confirm the good reception of the answer and I remain available for any further question,

Thank you and best regards

Celia MARTIN, Ph.D
Regulatory Affairs Director
LALLEMAND BIO-INGREDIENTS
Cell : + 34 645 134 980 |cmartin@lallemand.com |www.bio-lallemand.com

From: Overbey, Katie <Katie.Overbey@fda.hhs.gov>
Sent: Friday, June 7, 2024 4:46 PM

To: Martin Celia <cmartin@lallemand.com>
Subject: GRN 1154 - Additional Question

Dear Dr. Martin,
During our review of GRAS Notice 001154, we identified an additional question that needs to be addressed. Please find the question below:

1. In the amendment from April 17, 2024, Lallemand stated that their specification for lead for the lipase enzyme preparation is aligned with the Food Chemical Codex (13th edition, FCC 13) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA 2006). The results from three batch analyses for lead presented in GRN 1154 are two orders of magnitude lower compared to the FCC13 and JECFA specification of <5 mg/kg. Please re-consider reducing the specification for lead to reflect the batch analyses presented in Table 2.

We ask that you format your response such that each answer immediately follows the stated question and that you submit responses as a PDF or a response to this email. Please ensure that your responses do not contain confidential business information and please do not submit a revised version of the GRAS notice.

We respectfully request a response to these questions within 10 business days. If you are unable to complete the response within that time frame, please contact me to discuss further options.

Thank you in advance for your attention to our comments.

Best,
Katie

Katie Overbey, Ph.D., M.S (she/her/hers)
Regulatory Review Scientist

Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
Tel: 240-402-7536
katie.overbey@fda.hhs.gov


I GOT MY
COVID-19
VACCINATION