Application to amend Schedule 18 of the Australian New Zealand Food Standards Code to include a lipase enzyme from a modified strain of *Komagataella phaffii* as a Processing Aid

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3.1.1 General Requirements

B Applicant Details

(a) Applicant

Danstar Ferment AG, and affiliate of Lallemand Inc.

(b) Name of Contact persons responsible for the dossier

[redacted]

(c) Address

Poststrasse 30, CH-6300 Zug
Switzerland

(d) Telephone Number

[redacted]

(e) Email address

[redacted]

(f) Nature of applicant's business

Danstar Ferment AG is an affiliate of Lallemand Inc., a family-owned business headquartered in Canada whose focus is on the development, production, and marketing of yeasts, bacteria, fungi and enzyme solutions across the baking, food ingredients, human and animal nutrition and health, crop protection/nutrition, oenology, brewing, alcohol and biofuel sectors.

(g) Details of other individuals, companies or organisations associated with the application

No other individuals, companies or organisations are associated with this application.

C Purpose of the Application

The purpose of this application is to request for the addition to Schedule 18 of the Australia New Zealand Food Standards Code of a lipase enzyme (triacylglycerol lipase) produced from a *Komagataella phaffii* strain (LALL-LI2) engineered to express the native lipase gene from *Fusarium oxysporum*.
Lipases (EC 3.1.1.3) from various sources are already listed as permitted enzymes in Schedule 18.
The lipase enzyme subject to this application is not protein engineered.

D Justification for the Application

Lipases (EC 3.1.1.3) from various sources are already listed as permitted enzymes in Schedule 18. This application is for adding *Komagataella phaffii* containing the lipase gene from *Fusarium oxysporum* as a new source organism for lipase.

The amino acid sequence of the enzyme from this source is 100% homologous to the native lipase enzyme from *Fusarium oxysporum* (see Appendix 15 - Source of the Food Enzyme (CONFIDENTIAL) for the amino acid sequence).

D.1 Regulatory Impact Information

D.1.1 Costs and Benefits of the Application

(a) Cost and benefit to consumers

It is not anticipated that the inclusion of this enzyme processing aid in Schedule 18 will have any cost impact on consumers. Our application relates to an equivalent enzyme, in terms of technological effect in baking, to an enzyme already listed in Schedule 18, but expressed in another microorganism. In fact, lipases (EC 3.1.1.3) from various sources are already listed as permitted enzymes in Schedule 18. The addition of the enzyme described in the dossier provides an alternative source of lipase.

(b) Cost and benefit to industry and business

Our application relates to an equivalent enzyme, in terms of technological effect in baking, to an enzyme already listed in Schedule 18, but expressed in another microorganism. In fact, lipases (EC 3.1.1.3) from various sources are already listed as permitted enzymes in Schedule 18.

Nevertheless, the inclusion of lipase from another source will provide industry with an alternative option and hence more competition. Additionally, the use this particular lipase would be beneficial as high productivity is obtained with the strain *Komagataella phaffii* LALL-LI2, leading to cost benefit for the final enzyme user.

Therefore, as compared to the current situation, addition of the lipase from LALL-LI2 as a permitted enzyme in Schedule 18 will have the double benefit to increase the choice for local industry and to participate to the reduction of production cost.

(c) Cost and benefit to government

The inclusion of this enzyme processing aid in Schedule 18 will not have any cost impact on government. Our application relates to an equivalent enzyme, in terms of technological effect in baking, to an enzyme already listed in Schedule 18, but expressed in another microorganism. In fact, lipases (EC 3.1.1.3) from various sources are already listed as permitted enzymes in Schedule 18.

D.1.2 Impact on International Trade

The inclusion of lipase enzyme produced from a *Komagataella phaffii* strain engineered to express the native lipase gene from *Fusarium oxysporum* in the Australia New Zealand Food Standards Code as a processing aid may promote international trade on products using this enzyme as a processing aid, and reduce technical barriers to trade.

E Information to Support the Application

This application is based on Chapter 3.3.2 (Guidelines for applications for substances added to food – Processing aids) of the Food Standards Australia New Zealand Application Handbook. It relates to an enzyme processing aid.

E.1 Data Requirements

E.1.1 Data related to safety Studies

Please refer to Section 3.3.2 Processing Aids, C Information Related to the Safety of an Enzyme Processing Aid.

E.1.2 Data related to surveys on chemicals and other substances in food

Please refer to section 3.3.2 Processing Aids, F Information Related to Dietary Exposure of the Processing Aid.

E.1.3 Data related to epidemiological / intervention studies in human

No data resulting from epidemiological or intervention studies in human is provided to support this application.

F Assessment Procedure

The applicant considers the appropriate assessment procedure for the application to add lipase produced from *Komagataella phaffii* containing the native gene isolated from *Fusarium oxysporum* coding for lipase to Schedule 18 of the Australia New Zealand Food Standards Code to be the General Procedure, Level 1 (maximum 240 variable hours). This is based on the fact that lipases (EC 3.1.1.3) from various sources are already listed as permitted enzymes in Schedule 18. This application is for a new source organism of lipase.

G Confidential Commercial Information (CCI)

The following sections of the dossier contain information that is claimed confidential in this submission:
Appendix 3 - Technological effect of the Enzyme Processing Aid
Appendix 4 - Lipase Activity Determination Method
Appendix 6 - Manufacturing Process – List of Raw Materials and Processing Aids
Appendix 11 - Determination of Absence of Production Strain
Appendix 13 - Dynamic Upper GI Simulation Report Lipase from LALL-LI
Appendix 14 - AllergenOnline Search
Appendix 15 - Source of the Food Enzymes
Appendix 16 - Whole Genome Sequencing Analysis Report
Appendix 17 - FastQC Reports

These sections contain information related to the genetic engineering of the strain used to produce the lipase processing aid, the production process of the enzyme processing aid, and its technological effect. They regarded by Danstar Ferment AG as Confidential Commercial Information and are provided in the application strictly on this basis.

Danstar Ferment AG has expended a considerable amount of resources on long-term screening, research and development which has and continues to enable Danstar Ferment AG competitiveness. The lipase production strain and the way it was genetically engineered, as well as data on the lipase manufacturing process and its technological effect is part of the intellectual property, more specifically confidential technical and scientific know-how of Danstar Ferment AG (also herein referred as trade secrets).
This information is not only relevant to the lipase subject to this application, but also to other enzymes and/or productions strains developed for completely unrelated purposes. As such, disclosing publicly such information would necessarily harm Danstar Ferment AG competitiveness, not only in its enzyme business, but in other unrelated businesses. Because this information is sensitive and must remain confidential, Danstar Ferment AG personnel involved in the genetic engineering and characterization of yeast strains are bound by a confidential agreement and are contractually obliged not to disclose such information while being employed at Danstar Ferment AG and after having left the company. Furthermore, for the same reasons, the information is not included in our publications, including our patent applications, so as to prolong the confidentiality of this information.
The disclosure of these information to competition would lead to a loss of intellectual property, financial rewards and competitive advantage belonging to Danstar Ferment AG In fact, disclosing related information judged as confidential, would allow competitors to develop similar products without the same expenditure of resources.

Maintaining this information as confidential is therefore required to reduce the likelihood of a competitor manufacturing a similar product without investing resources (economic, human, time) in conducting the necessary research and development required to develop such a product.

Consequently, the disclosure of this confidential information would be expected to cause significant harm to the Danstar Ferment AG competitive position and could result in a material financial loss and a material financial gain to its competitors. If this confidential information was made known, competitors would require significantly less capital investment to duplicate this organism/enzyme, thereby allowing competitors to realize a profit in much less time than could Danstar Ferment AG. Consequently, this would confer to Danstar Ferment AG a severe competitive disadvantage in the marketplace.

A summary of the confidential information is presented under Section 3.3.2 of this dossier.

H Other Confidential Information

Not applicable.

I Exclusive Capturable Commercial Benefit (ECCB)

Danstar Ferment AG is not claiming ECCB. This application relates to an equivalent enzyme, in terms of technological effect in baking, to an enzyme already listed in Schedule 18, but expressed in another microorganism. In fact, lipases (EC 3.1.1.3) from various sources are already listed as permitted enzymes in Schedule 18. The inclusion of lipase from another source will provide industry with an alternative option and hence more competition.

J International and Other National Standards

J.1 International Standards

Lipase from *Fusarium oxysporum* produced by *Komagataella phaffii* has not been reviewed by JECFA; there is no specific Codex Standard relevant to this application.

J.2 Other National Standards or Regulations

Please find below the status of submission to other countries for the lipase from LALL-LI2, as of April 8, 2024.

United States:

A Generally Recognized as Safe (GRAS) notice was submitted, and filed by the FDA on November 16, 2023 as GRN 1154. The notice is currently under evaluation by the FDA, and the current status is pending[1].

European Union:

While a union list of authorised food enzymes has not been published yet in the European Union, an application for authorisation of the enzyme processing aid has been submitted on January 18, 2024 to the European Commission (EC) for evaluation by the European Food Safety Agency Panel on Food Contact Materials, Enzymes and Processing Aids (EFSA CEP Panel). The application has been considered valid by the EC and is currently under suitability/completeness check by EFSA, under EFSA-Q-2024-00201[2].

Canada:

An application for the approval of the enzyme processing aid for use as a food additive (food enzyme) in Canada has been submitted 0n September 18, 2023 2023 to Health Canada and is currently under review.

K Statutory Declaration

Please see Appendix 1 - Statutory Declaration.

L Checklists

This application concerns an enzyme product intended to be used as a processing aid. Therefore, the applicable checklists are:

- Section 3.1.1 – General requirements
- Section 3.3.2 – Processing aids, subsections A, C, D, E, F

Checklist can be found in Appendix 2 - Checklists.

[1] https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=1154
[2] https://open.efsa.europa.eu/questions/EFSA-Q-2024-00201

3.3.2 Processing Aids

A. Technical Information on the Processing Aid

A.1 Information on the type of processing aid

The processing aid subjects to this application is a lipase enzyme produced from a *Komagataella phaffii* strain with a safe history of use, that has been engineered to express the native lipase gene from *Fusarium oxysporum*. The obtained production strain is referred to as *Komagataella phaffii* LALL-LI2, *K. phaffii* LALL-LI2, or LALL-LI2.
This lipase is not protein engineered.

A list of already permitted enzymes is available in the Australia New Zealand Food Standards Code, Schedule 18 (FSANZ, processing aids), under section 18-4 (permitted enzymes). Permitted enzymes of microbial origin are listed under subsection 5. Lipase, triacylglycerol (EC 3.1.1.3) from various sources is listed as a permitted enzyme.

A.2 Information on the identity of the processing aid:

IUBMB Name	Triacylglycerol lipase
Common/Accepted names	Lipase, triglyceride lipase, glycerol ester hydrolase, tributyrase, butyrinase, tributyrinase, tributyrin esterase, triglyceride hydrolase; triglyceridase; triacylglycerol ester hydrolase
Systematic name	Triacylglycerol acylhydrolase
IUBMB No	EC 3.1.1.3
CAS registry No	9001-62-1

The lipase enzyme assessed in this dossier is produced by a strain of *Komagataella phaffii* expressing the native lipase gene from *Fusarium oxysporum*. Therefore, this enzyme has not been protein engineered.

Information on the Recipient (Host) Organism

The host strain used for construction of the production strain LALL-LI2 is *Komagataella phaffii* ATCC 76273, also known as NRRL Y-11430 and CBS 7435, and an isolate colony identified as M17500 was used. 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 *Komagataella phaffii* following multigene sequence analyses (Kurtzman *et al*. 2009).

K. phaffii has an extensive history of use in the food and feed industry and has been utilized for many years for production of single-cell protein and enzymes (Spohner *et al*., 2015; Barone et al., 2023).
Komagataella phaffii NRRL Y-11430 has a long history of safe use as a production organism in the food industry (Offei *et al*. 2022).

The taxonomic classification is presented in Table 1 below.

Kingdom	Fungi
Division	Ascomycota
Class	Saccharomycetes
Order	Saccharomycetales
Family	*Phaffomycetaceae*
Genus	*Komagataella*
Species	*Komagataella phaffii* (previously *Pichia Pastoris*)
Strain	ATCC 76273 / M17500

Table 1: Taxonomic Classification of Host Strain

A *K. phaffii* strain derived from strain ATCC 76273 (see strain lineage in GRN 737 part 1, figure 2 page 13, U.S. FDA 2018) is an approved source organism for the production of soy leghemoglobin in Canada. The soy leghemoglobin preparation has 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[3].

Soy leghemoglobin is also an authorised food produced using gene technology in Australia/New-Zealand as per schedule 26[4], for use in a meat analogue products. In its risk and technical assessment report (see application A1186[5]), FSANZ mentioned that *Komagataella phaffii* has been classified as a Biosafety Level 1 organism, and the source strain, which is derived from strain ATCC 76273/NRRL Y-11430/CBS 7435 (see figure 1 in FSANZ report) 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. phaffii* and no reports of adverse effects from products produced from *K. phaffii* strains were identified by a literature search.
Moreover, three protein engineered enzymes sourced from *Pichia pastoris* (former designation of *Komagataella Phaffii*) are authorized as a processing aid in FSANZ schedule 18, for the conversion of purified stevia leaf extract to produce rebaudioside D, E, and M (FSANZ, 2023).

Additionally, phospholipase C enzyme (GRN 204, U.S. FDA 2006), 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 strain ATCC 76273 (see strain lineage in Figure 1 below), and these substances have received a no questions letter from the FDA.

[3] https://www.canada.ca/en/health-canada/services/food-nutrition/genetically-modified-foods-other-novel-foods/approved-products/soy-leghemoglobin.html
[4] https://www.legislation.gov.au/F2015L00450 [Last update Nov. 30, 2023]
[5] https://www.foodstandards.gov.au/food-standards-code/applications/A1186

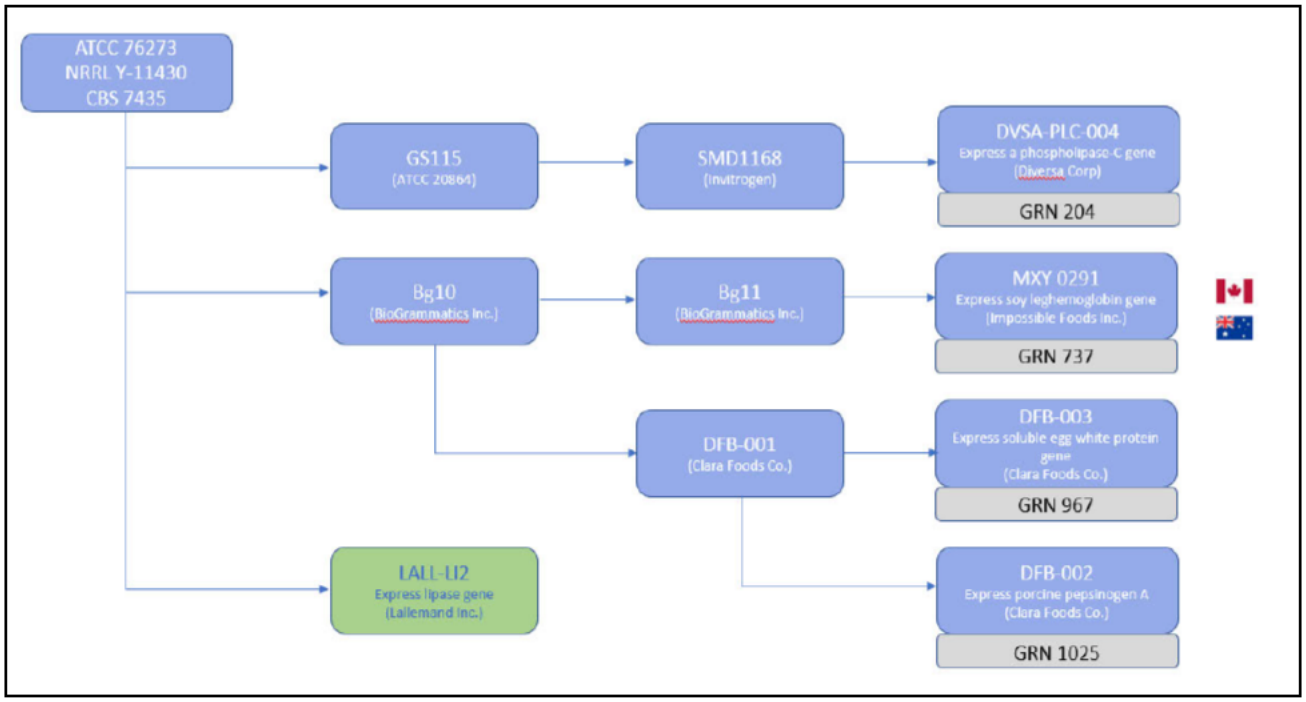

Figure 1: LALL-LI2 Strain Lineage

Finally, *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), which is the case for *K. phaffii* LALL-LI2, as shown by the data provided in this application.

Information on the Donor Organism

The donor of the lipase gene is Fusarium oxysporum. The taxonomic classification of the donor microorganism is presented in Table 2.

Kingdom	Fungi
Division	Ascomycota
Class	Sordariomycetes
Order	Hypocreales
Family	*Nectriaceae*
Genus	*Fusarium*
Species	*Fusarium oxysporum*
Strain	-

Table 2: Taxonomic classifications of host and donor microorganisms

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

Fusarium oxysporum is already listed as an accepted gene donor for both lipase and trypsin in the Australia New Zealand Food Standards Code, Schedule 18.

A.3 Information on the chemical and physical properties of the processing aid:

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.

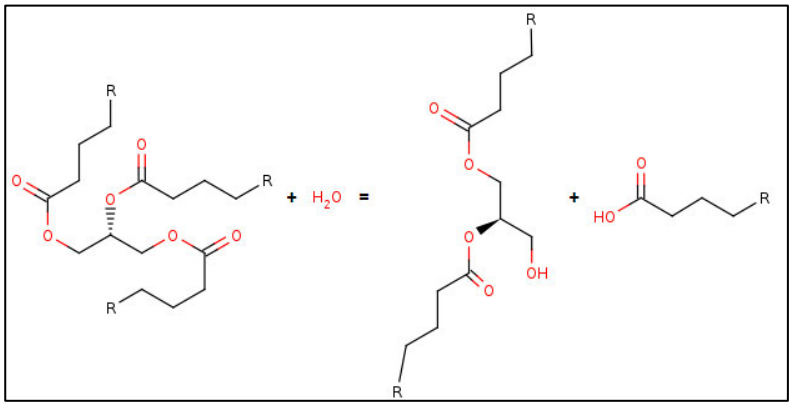

Figure 2: Enzymatic Reaction of Lipase6

Triglycerides are an important reservoir of stored energy in human body and derive primarily from animal fats and vegetable oils (Wang *et al*. 2013). They are a major component of human skin oil (Lampe *et al*., 1983). Triglycerides are also found in wheat flour: wheat flour contains approximately 2.0 -2.5% lipids (Gerits *et al*. 2014a), divided into polar (e.g., phospholipids) and non-polar lipids (e.g., triglycerides).

The enzyme is intended to be used as a processing aid in baking processes to improve dough structure and behavior during baking, increase bread volume and improve crumb structure.

Lipases, such as the one from LALL-LI2, can react with various lipid components in the dough thereby converting triglycerides into diglycerides, monoglycerides and free fatty acid. Also, lipases act on phospholipids and galactolipids converting them to more efficient emulsifying structures like lysophspholipids and digalactosyl monoglycerides (Gerits et al. 2014b). These lipase-generated emulsifiers (such as mono- and di-glycerides and lysophosphoilipids and digalactosyl monoglycerides) can interact with key dough components and enhance the dough stability and dough development as explained below:

- During the process of dough mixing, these emulsifying molecules (mono- and di-glycerides) can bind to lipophilic regions of gluten proteins. This interaction could result in enhanced gluten protein aggregation process imparting improved dough stability (Melis et al. 2017).

- Endogenous emulsifying lipids present in wheat flour and those molecules additionally generated by the action of the lipase (as mentioned above) on lipid substrates do affect starch gelatinization process and could exert extended oven spring resulting in increased loaf volume. These amphipathic emulsifiers can also form hydrogen bonds with gluten and starch molecules causing enhanced dough strengthening effects (Melis et al. 2017).

- An important aspect that decides the overall bread quality in terms of volume and crumb structure is the formation and abundance of tiny gas cells during the process of dough mixing. These gas cells

[6] Image from: https://www.brenda-enzymes.org/enzyme.php?ecno=3.1.1.3

should also withstand the subsequent bread fermentation process and need to be stabilized. The stabilization of these gas cells in the gluten starch matrix of dough is accomplished by dough stabilizing emulsifiers produced by the conversion of flour lipids by the lipase.

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): Improved emulsification results in better dispersion of all components in the dough system. In addition, lipases promote interactions between lipids, proteins, and starch, and these interactions are believed to be responsible for dough reinforcement. These above effects enhance the dough stability and enables better handling of the same (Dai & Tyl 2021).

- Improve the dough structure and behavior (how well a dough performs through proofing/fermentation and baking), resulting in consistent baking process: During the dough mixing process a uniform mixing of all dough components is desired, including gluten and starch, as well as the creation of tiny gas cells in the dough structure. The lipase-generated emulsifiers support those processes, and they further assist in stabilizing the gas cell through the proofing/fermentation and baking process.

- Contribute to enhance bread volume and shape: The molecules with enhanced emulsification potential discussed above will result in higher loaf volume, as well as a finer and tighter crumb structure with a whiter looking crumb color due to a better light reflection. Through gas cell stabilization emulsifier molecules can allow retention of significant carbon dioxide gas during the process of fermentation and baking. This results in the overall enhancement in bread volume. Cumulatively, a bread with significantly finer crumb structure and higher loaf volume can be, in general, regarded as “enhanced bread shape” (Melis and Delcour 2020).

The lipase processing aid 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. The optimum temperature and pH for the lipase food enzyme is 30 to 37°C and pH 5 to 7, respectively.
The enzyme is completely deactivated after 15 min at temperatures above 60 °C, and has no further technological effect after baking (See also section F.2 The levels of residues of the processing aid or its metabolites for each food or food group). Internal tests have demonstrated the technical effect of the enzyme processing aid (See Appendix 3 - Technological effect of the Enzyme Processing Aid (CONFIDENTIAL)).

The lipase food enzyme is specifically characterized by its lipase activity.
Carboxylic ester hydrolase activity of lipases results in hydrolysis of tributyrin (glyceryl tributyrate) generating butyric acid as one of the products. Lipase activity is determined using an internal method by monitoring formation of butyric acid through hydrolysis of emulsified tributyrin with gum arabic used as an emulsifier, by titration with NaOH solution as the titrant and comparison against an internal standard (Cf. Appendix 4 - Lipase Activity Determination Method (CONFIDENTIAL) for details of analytical method).
The enzyme activity is expressed in Lallemand Baking Lipase Units/g (LBLU/g). One LBLU is defined as the enzyme quantity that produces 1 micromole of butyric acid per minute at 21°C and pH=7.

A.4 Manufacturing Process

The lipase food enzyme is produced by fermentation of *Komagataella phaffii* LALL-LI2 and subsequent downstream processing.

The lipase food enzyme is manufactured at industrial scale 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.

The 3 batches submitted in this application were produced in Lallemand scale-down pilot laboratory. The process applied is representative of the industrial-scale process, mainly based on the following criteria (non-exhaustive list):
- Raw materials and processing aids are the same
- Fermentation recipes
- Fermentation temperature and pH
- Oxygen transfer rate
- Fermentation time
- Primary separation by centrifugation and application of equivalent wash conditions
- Clarification by cross flow microfiltration
- Concentration by cross flow ultrafiltration using equivalent membrane material and cut-off and similar concentration factors, scaled based on equivalent permeate flux. Operated targeting similar concentration factors and diafiltration volumes.
- Polish filtration by depth filtration using disposable filtration modules comprised of cellulose fibers, filter aids and resins.

A schematic overview of the manufacturing process for the lipase processing aid is presented in Appendix 5 - Manufacturing Process Flow Chart and all the steps are described in the following sections.
A list of the raw materials and processing aids used in the production of the lipase enzyme processing aid at is provided in Appendix 6 - Manufacturing Process – List of Raw Materials and Processing Aids (CONFIDENTIAL). All raw materials and processing aids used in the manufacture of the food enzyme are acceptable for use in the manufacture of food enzymes and are commonly used in food industrial processes.

Fermentation Process

i) 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.

ii) Pure Culture Stage 2 and Feb-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 range for the fermentation temperature is 30-34°C while the one for the pH is 4.5-6.0. 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.

The genotypic stability of the production strain during the propagation procedure has been demonstrated by PCR genotyping comparison of DNA isolated from the cells used for seeding the yeast propagation, and from the final yeast cream, for the 3 batches described in this application. (Cf. section D.3 Information on the genetic stability of the source microorganism).

Recovery and Formulation of the Enzyme Processing Aid (Downstream Processing)

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

Finally, the enzyme is formulated to obtain the food enzyme preparation.

i) Primary Solid/Liquid Separation

The purpose of the primary separation is to free the soluble fraction containing the enzyme from the yeast cells. To minimize loss of enzyme activity the separation is performed at a defined pH and a specific temperature range. For the 3 batches described in the dossier, separation was performed by continuous centrifugation. Nevertheless, depending on the scale of the process and the site of operation, separation may either also be conducted by filtration.
The yeast cells are then washed with water to ensure optimal enzyme recovery. The duration of the separation step is defined by the initial volume to be separated, the throughput of the separators (centrifugation or filtration) and the wash factor applied.
At this point 1.5 g/L of sodium chloride is added to the liquid fraction to increase ionic strength and stabilize the enzyme extract.

ii) Clarification

Most residual yeast cells remaining after primary separation were removed by cross flow microfiltration on tubular ceramic membranes using a cut-off small enough to retain the *K. phaffii* cells in the retentate. Alternatively, to cross flow microfiltration, 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.

iii) Enzyme Purification and Concentration

At this point, the overall extract is diluted (around 5% solids) due to the applied wash volumes during primary separation. Increase of enzyme purity and solid content is achieved by ultrafiltration using a molecular weight cut off (MWCO) large enough to remove smaller amino acids and peptides, but smaller than the enzyme. The use of ultrafiltration will also improve the ratio enzyme activity/TOS. Temperature and pH are controlled to minimize the loss of enzymatic activity, which is performed until the desired concentration has been obtained.

iv) Polish Filtration

Polish filtration is applied at the end of the recovery process to remove insoluble substances such as precipitants produced during the previous step, any remaining residual yeast cells, and microbial contaminants. The approximate filters' cut-off is ranging between 0.1 to 3 μm, depending on the disposable filter cartridge used. Specifically for the 3 batches described in this dossier, two filtration steps using membranes with retention rating of 0.1 to 0.3 μm were performed. The filters used are composed of cellulosic fibers, diatomaceous earth and resins which enables them remove contaminants based on both size exclusion and electrokinetic adsorption.

This final polish filtration at the end of the recovery process results in a concentrated enzyme solution free of the production strain and insoluble substances.

v) Formulation and Packaging

The purpose of this step is to formulate the enzyme as a dried solid product. The resulting product is defined as a food enzyme preparation.

Drying of the enzyme extract is performed by spray-drying. Food-grade maltodextrin is added to the liquid extract to increase solid content in the final liquid before drying, improve enzyme stability during drying, and standardize enzyme concentration in the food enzyme preparation. The drying process is operated at specific conditions (e.g., inlet / outlet temperatures) such as to prevent the enzyme from thermal inactivation and to control powder characteristics (such as water content, particle size, dustiness, density).

The food enzyme preparation is tested by Quality Control for all quality related aspects before release, including expected enzyme activity and the general JECFA Specification for Food Enzyme Preparations.

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.

Quality Control

As mentioned above, the lipase is produced at certified facilities, following established procedures, in accordance with cGMP for food and the principles of HACCP.
Routine testing is conducted to confirm that the manufactured food enzyme preparation is of food-grade quality and meets international standards/specifications for food enzymes (see Section 2.5). To ensure that the food enzyme preparation meets these quality criteria, potential hazards are taken into account and controlled during the whole production process as described below.

i) Microbiological Hygiene

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

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. The whole process is computer controlled, which reduces the probability of human errors in critical process steps.
These in-process controls include, but may not be limited to:

- Microbial controls: Absence of significant microbial contamination is 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.

A.5 Specification for Identity and Purity

Food-grade specifications for the lipase are presented in Table 3 below. The specifications for the food enzyme comply with the current purity and microbial limits established for enzyme preparations by JECFA (2006) and in the FCC (FCC, 2023). For arsenic, cadmium, and mercury, not included in JECFA or FCC, specifications are equivalent or lower than those in FSANZ Schedule 19, section S3-4[7].

[7] https://www.legislation.gov.au/F2015L00493/latest/versions

As described above, the lipase food enzyme from *K. phaffii* LALL-LI2 is manufactured as a dried powder in a standardized format.

Parameter	Specification	Method of Analysis
Enzyme activity		
Lipase activity (LBLU/g)	> 10,000	Internal Method
Microbiological Parameters		
Total aerobic plate count (CFU/g)	≤ 50,000	Internal Method
Coliforms (CFU/g)	≤ 30	Internal Method
Escherichia coli (/25g)	Not Detected	ISO 16649-3
Salmonella (/25g)	Not Detected	NEOGEN® ANSR kit for Salmonella (AOAC certified)
Antibacterial activity	Absent	Following JECFA guidelines[8]
Production Organism (/g)	Absent	Following EFSA guidelines[9]
Heavy Metals		
Lead (ppm)	≤ 5	AOAC 2011.14
Arsenic (ppm)	≤ 1	AOAC 2011.14
Cadmium (ppm)	≤ 0.5	AOAC 2011.14
Mercury (ppm)	≤ 0.5	AOAC 2011.14

CFU = colony forming units; LBLU = Lallemand Baking Lipase Unit; ppm = parts per million.

Table 3: Product Specifications for Lipase from *Komagataella phaffii* LALL-LI2

Danstar Ferment AG confirms that lipase processing aid is free from known allergen. In particular, the glucose used in the fermentation process is not source from wheat and maltodextrin used for the formulation is sourced from corn syrup.

Certificates of analyses for these 3 independent pilot batches of the enzyme processing aid are provided in Appendix 7 - Certificates of Analysis. Specification parameters are analysed using internationally recognized analytical methods or appropriate internal methods.

Internal Methods:

Enzyme activity:
As described in section A3, lipase activity is determined using an internal method by monitoring formation of butyric acid through hydrolysis of emulsified tributyrin with gum Arabic used as an emulsifier, by titration with NaOH solution as the titrant and comparison against an internal standard.
The method provided in Appendix 4 - Lipase Activity Determination Method (CONFIDENTIAL).

[8] 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
[9] 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

Microbiological parameters:
The internal method for total plate count is provided in Appendix 8 - Total Plate Count Method, and for coliforms in Appendix 9 - Coliforms Method.

Antibacterial activity:
The antibacterial activity method is provided in Appendix 10 - Antibacterial Activity Method.

Production organism:
The method used to determine the absence of production organism is provided in Appendix 11 - Determination of absence of production strain (CONFIDENTIAL).

A.6 Analytical Method for Detection

This information is not required in the case of an enzymatic processing aid.

B Information Related to the Safety of a Chemical Processing Aid

This section is not applicable as the processing aid subject to this application is an enzyme.

C Information Related to the Safety of an Enzyme Processing Aid

C.1 General information on the use of the enzyme as a food processing aid in other countries

The lipase from *K. phaffii* LALL-LI2 has not been yet permitted in other international jurisdictions for the uses subject to the current application.
It has been submitted for evaluation in the USA, the EU, and Canada. Please find below the status of submission, as of April 8, 2024.

United States:
A Generally Recognized as Safe (GRAS) notice was submitted, and filed by the FDA on November 16, 2023 as GRN 1154. The notice is currently under evaluation by the FDA, and the current status is pending[1].

European Union:
While a union list of authorised food enzymes has not been published yet in the European Union, an application for authorisation of the enzyme processing aid has been submitted on January 18, 2024 to the European Commission (EC) for evaluation by the European Food Safety Agency Panel on Food Contact Materials, Enzymes and Processing Aids (EFSA CEP Panel). The application has been considered valid by the EC and is currently under suitability/completeness check by EFSA, under EFSA-Q-2024-00201[2].

Canada:
An application for the approval of the enzyme processing aid for use as a food additive (food enzyme) in Canada has been submitted 0n September 18, 2023 2023 to Health Canada and is currently under review.

Additionally, the lipase from *S. cerevisiae* LALL-LI, also developed by Danstar Ferment AG/Lallemand Inc. and corresponding to the same native lipase protein from *F. oxysporum* but produced from a different microorganism, has been positively evaluated or permitted in various areas, including:

- Canada, for use in bread, flour, whole wheat flour and unstandardized baking products (Health Canada, 2023).
- EU, for use in baking processes (EFSA CEP Panel, 2023).
- USA, in the manufacture of baked goods (GRN 1047, U.S. FDA 2023b). Please note that a no questions letter was received from the FDA on May 12, 2023. At the time of submission of the current application this letter has not been yet made available on the FDA website. It may be provided to FSANZ upon request.

Finally, the lipase protein from *K. phaffii* LALL-LI2 is also closely related to the following lipase proteins from *Fusarium oxysporum*, which have been evaluated as safe, either by the US Food and Drug Administration (FDA), the European Food Safety Authority (EFSA), the Food Standards Australia New Zealand (FSANZ), and/or Health Canada:

- A lipase produced by *Aspergillus oryzae* MStr110 expressing the gene encoding a lipase from *Fusarium oxysporum*: This enzyme, has been considered as GRAS by the FDA (GRN 075, U.S. FDA 2001). This enzyme has also been evaluated by FSANZ (FSANZ, 2002) and is included in the list of enzymes authorised to be used as a processing aid in Australia and New-Zealand (FSANZ, 2023).
- A lipase produced by *Trichoderma reesei* RF10625 expressing the gene encoding a lipase from *Fusarium oxysporum*: This enzyme, has been considered as GRAS by the FDA (GRN 631, US FDA 2016). This enzyme has also been evaluated by FSANZ (FSANZ, 2019) and is included in the list of enzymes authorized to be used as a processing aid in Australia and New-Zealand (FSANZ 2023). It is also included in the list of permitted food enzymes in Canada (Health Canada, 2023), Additionally, it has been evaluated as safe by EFSA (EFSA CEP Panel 2019).

C.2 Information on the potential toxicity of the enzyme processing aid

C.2.1 Information on the enzyme's prior history of human consumption and its similarity to proteins with a history of safe human consumption

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

Regulatory Approvals/Safety Evaluations

Moreover, extensive regulatory approvals or safety evaluations support the safety of lipase enzymes from various microorganisms, including FDA, JECFA, Food standards Australia New Zealand (FSANZ), Health Canada and European Food Safety Authority (EFSA):

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 1047; U.S. FDA, 2023b).

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)[10].

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.*

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

oxysporum, and *T. reesei* containing the lipase gene from *Aspergillus tubingensis* are permitted enzymes in Schedule 18 of the Australia New Zealand Food Standards Code (FSANZ, 2023).

Health Canada

Various lipases from *Aspergillus niger*, *Aspergillus oryzae*, *Rhizopus oryzae*, *Saccharomyces cerevisiae*, *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 (Health Canada, 2023).

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) (EFSA CEP Panel, 2019)
- 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
- 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 (EFSA CEP Panel, 2023). This lipase corresponds to the enzyme protein subject to the current application, 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
- Triacylglycerol lipase from non-genetically modified *Limtongozyma cylindracea* strain MS-5-OF. https://www.efsa.europa.eu/en/efsajournal/pub/8256
- Triacylglycerol lipase from the genetically modified *Aspergillus luchuensis* strain FL105SC. https://www.efsa.europa.eu/en/efsajournal/pub/8259
- Triacylglycerol lipase from the genetically modified *Aspergillus luchuensis* strain FL108SC. https://www.efsa.europa.eu/en/efsajournal/pub/8260

C.2.2 Information on any significant similarity between the amino acid sequence of the enzyme and that of known protein toxins.

A bioinformatics search for similarity of lipase to known toxins was performed. A custom FASTA database of known toxins (provided as 'Annex 1 - Uniprot_db_keyword-toxin_2023-05-17' to this application) 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 (Search process detailed in Appendix 12 - Step-by-step process for toxin search).
There were two hits (see Table 4 below), both of which cover less than 30% of the lipase query sequence (only 82 residues and 93 residues, respectively) and both of which share less than 40% sequence identity with the query sequence across that interval. This is below the threshold (at least 80% amino acid similarity and 70% coverage of the query sequence) 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.

Accession ID	Specification	E-Value	Query Coverage	Pairwise Identity
A0A1F3WZY0_9PROT	Uncharacterized protein OS=Betaproteobacteria bacterium RBG_16_56_24 OX=1797475 GN=A2V79_10845 PE=4 SV=1	1.30e-02	25%	35%
A0A255ZH58_9BURK	Cadherin domain-containing protein OS=Rhodoferax sp. TH121 OX=2022803 GN=CHU94_07110 PE=4 SV=1	1.24e-03	28%	34%

Table 4: BLAST results for amino acid sequence of lipase from LALL-LI2

C.2.3 Information on the stability of the enzyme to degradation in appropriate gastric and, if applicable, intestinal model digestion systems.

The lipase enzyme from *S. cerevisiae* LALL-LI, which is, as already mentioned in section C.1, the same protein as the one from *K. phaffii* LALL-LI2 and has been positively evaluated by various authorities, has been tested in a simulated gastric digestion assay mimicking the conditions normally found in the human gastrointestinal system. The assay was conducted using a Simulator of the Human Microbial System (SHIME®) as described in Van de Wiele *et al*. (2015).
The assay was conducted in triplicate, simulating the upper gastrointestinal tract under fed conditions. The test article was administered at 350 mg/reactor at the beginning of the gastric phase, a quantity that simulates a dose of 5 mg powdered enzyme/kg bw/day, or 0.625 mg TOS/kg bw/day for a 70 kg person. The gastric phase involved incubation with stirring for 2h at 37°C, in a reactor in which a sigmoidal decrease of pH was carried out from 5.5 to 2.0, and to which pepsin, phosphatidylcholine, nutritional medium and salts

were added. The contents of the reactor were sampled at 0 and 120 min. After 2 h the small intestinal phase was initiated with increased pH from 2.0 to 5.5 within 5 min, from 5.5 to 6.5 in the first hour, 6.5 to 7 in the second hour and maintained at a constant 7.0 for a third hour. Pancreatic enzyme release was simulated by addition of trypsin and chymotrypsin (no pancreatin was used due to elution close to the lipase from LALL-LI), and bile release was simulated by addition of bovine bile extract. Sampling was conducted at 60, 120 and 180 min of the small intestinal phase.
Prior to conducting the assay, the separation of the relevant proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted. The enzyme eluted as a single band just above 25 kDa. It was degraded if spiked into stomach medium which contained pepsin, but no other components of the stomach or small intestinal media interfered with the elution of the enzyme.
Under the conditions of the assay, the enzyme was completely degraded at the 120 min (2 h) sampling time-point of the gastric phase, and therefore could not be detected at any sampling time-point of the small intestinal phase. The study report is provided in Appendix 13 - Dynamic Upper GI Simulation Report Lipase from LALL-LI (CONFIDENTIAL).
We consider these data as relevant to demonstrate that the lipase from LALL-LI2 would be degraded in the stomach during digestion.

C.2.4 Acute or short-term oral toxicity studies in a rodent species

It is considered that based on the supporting information provided in this application and on the qualifying parameters described below, the toxicological package including animal toxicity studies is not required in order to further demonstrate the safety of the lipase food enzyme from *K. phaffii* LALL-LI2 and can therefore be waived.

- The host strain belongs to *Komagataella phaffii* species, which has a long history of safe use and is included in the list of organisms considered suitable for Qualified Presumption of Safety (QPS) approach for safety assessment by EFSA, with the qualification that it applies for production purposes and no viable cells are present in the final product (EFSA BIOHAZ Panel, 2024). Data in this application demonstrate that no viable cells of the production strain remain in the product.

- The host strain *Komagataella phaffii* ATCC 76273/NRRL Y-11430/CBS 7435 has a long history of safe use as a production organism in the food industry (Offei *et al*. 2022). Additionally, FSANZ acknowledged that this strain has a recognised safe history of use for the production of food enzymes and is neither pathogenic nor toxigenic[5].

- In its scientific guidance for the submission of dossiers on food enzymes (EFSA CEP Panel, 2021), EFSA acknowledged that genetically modified organisms used for the production of food enzymes, that are derived from QPS host strains and for which no concerns are raised by the genetic modification, can be considered as QPS. LALL-LI2 has been confirmed to belong to the same species as the host strain by whole genome identification (see Appendix 16 - Whole Genome Sequencing Analysis Report (CONFIDENTIAL)) and the genetic modification is well characterized and safe (see Appendix 15 - Source of the Food Enzyme (CONFIDENTIAL). Therefore, LALL-LI2 can also be considered as a strain with QPS status.

- The production strain LALL-LI2 is constructed via linear DNA transformation with synthetic genes to avoid any unintended transfer of genetic elements from the donor strain to the host strain. Thus, the modified yeast contains only a limited introduced sequence pertaining to the gene of interest. This has been confirmed

by Whole-Genome Sequencing (WGS) analysis (see Appendix 16 - Whole Genome Sequencing Analysis Report (CONFIDENTIAL)).

-The WGS analysis of the production strain LALL-LI2 has also demonstrated the absence of genes of potential concern.

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

- The lipase protein from *Fusarium oxysporum*, expressed in *Saccharomyces cerevisiae* LALL-LI, which is the same as the one expressed in *Komagataella phaffii* LALL-LI2, has been evaluated a safe by various authorities (see section C.1).

- The absence of viable cells from the production strain were confirmed in 3 batches of the lipase food enzyme and is part of the enzyme specifications.

Therefore, product specific toxicity studies have not been conducted with the lipase from *K. phaffii* LALL-LI2.

C.3 Information on the potential allergenicity of the enzyme processing aid

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 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[11] 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 (see Appendix 15 - Source of the Food Enzyme (CONFIDENTIAL) for the 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 sequence homology search was also conducted using the exact 8-mer approach, which is considered to be highly conservative, and did not identify any matches.
The original reports obtained on AllergenOnline are provided in Appendix 14 - AllergenOnline Search (CONFIDENTIAL).

The lipase protein from *Fusarium oxysporum*, expressed in *Saccharomyces cerevisiae* LALL-LI, which is the same as the one expressed in *Komagataella phaffii* LALL-LI2, has been evaluated a safe by various authorities. Notably, in its scientific opinion, EFSA mentioned that the likelihood of allergic reactions upon dietary exposure to this protein is low (EFSA CEP Panel, 2023).

Based on the information provided above, no evidence exists that might indicate that the lipase from LALL-LI2 would produce an allergenic response following consumption of foods to which the enzyme is added.
A search of the available scientific literature did not reveal any evidence indicating allergenicity to lipase in consumers of foods to which the enzyme is added.
Furthermore, any residual enzyme carried over into the final ingredient would likely be inactivated and denatured under the conditions of food processing during production of the final food products.

Also, the lipase processing aid does not contain any major food allergens from the fermentation media: Yeast extract, which could potentially be considered as a source of allergen, is utilized by the production stain during fermentation, and the yeast biomass and fermentation solids are removed during downstream processing. Additionally, glucose is not sourced from wheat.
The final formulation components do not include or originate from sources that are major food allergens. In particular, the maltodextrin used as a carrier is sourced from corn syrup.

Taken all the above into consideration, the use of the lipase enzyme is not anticipated to pose any allergenicity concerns in consumers.

C.4 Safety assessment reports prepared by international agencies or other national government agencies, if available

Please refer to section C.1.

[11] AllergenOnline is an allergen protein database containing 2,233 peer-reviewed allergenic protein sequences 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/

D Additional Information Related to the Safety of an Enzyme Processing Aid Derived from a Microorganism

D.1 Information on the source microorganism

The source organism (production strain) is obtained by genetic engineering of a *Komagataella* phaffii strain (ATCC 76273) with a safe history of food use (see sectionA.2), that has been engineered to express the native lipase gene from Fusarium oxysporum.

The host strain *K. phaffii* ATCC 76273 is available and was sourced from the American Type Culture Collection (ATCC)[12]. An isolate colony of the strain ATCC 76273, identified as M17500 was used for the strain engineering.

The genome of the production strain has been sequenced and analyzed (see in Appendix 16 - Whole Genome Sequencing Analysis Report (CONFIDENTIAL) and Appendix 17 - FastQC Reports (CONFIDENTIAL)).
The production strain has been confirmed to be a *Komagataella phaffii* strain and contains only a limited introduced sequence pertaining to the gene of interest (see Appendix 16 - Whole Genome Sequencing Analysis Report (CONFIDENTIAL)).

It is customary to use the Pariza-Johnson decision tree (Pariza and Johnson, 2001) to evaluate the safety of modified strains for enzyme production. The analysis includes, but is not limited to, the identity of the parental strain, characteristics of the introduced DNA (the sources and functions of the introduced genetic material), an outline of the genetic construction of the production strain, a characterization of the production strain, and potential for the production strain to have pathogenic, toxigenic or antibiotic resistance characteristics. If the production microorganism meets the criteria described by Pariza and Johnson, it can be determined safe as used for food production.

Pariza and Johnson base the decision tree concept on their 1983 publication (Pariza and Foster, 1983) 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 recombinant DNA 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 recombinant DNA strain improvement technologies.” (Pariza and 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 and Johnson, 2001).

[12] https://www.atcc.org/products/76273

The safety of the production strain and consequently the one of the lipase food enzyme was assessed using the Pariza and Johnson decision tree (see Figure 3 below). The production strain is genetically modified using standard recombinant DNA techniques, and the gene is integrated into designated loci of the *K. phaffii* parental strain. The production strain is free of transferable antibiotic resistance gene DNA. The introduced DNA is well-characterized and free of attributes that would render it unsafe for use in food products, such as bread. Based on this approach, the lipase food enzyme derived from genetically modified *K. phaffii* LALL-LI2 is accepted and suitable for food production.

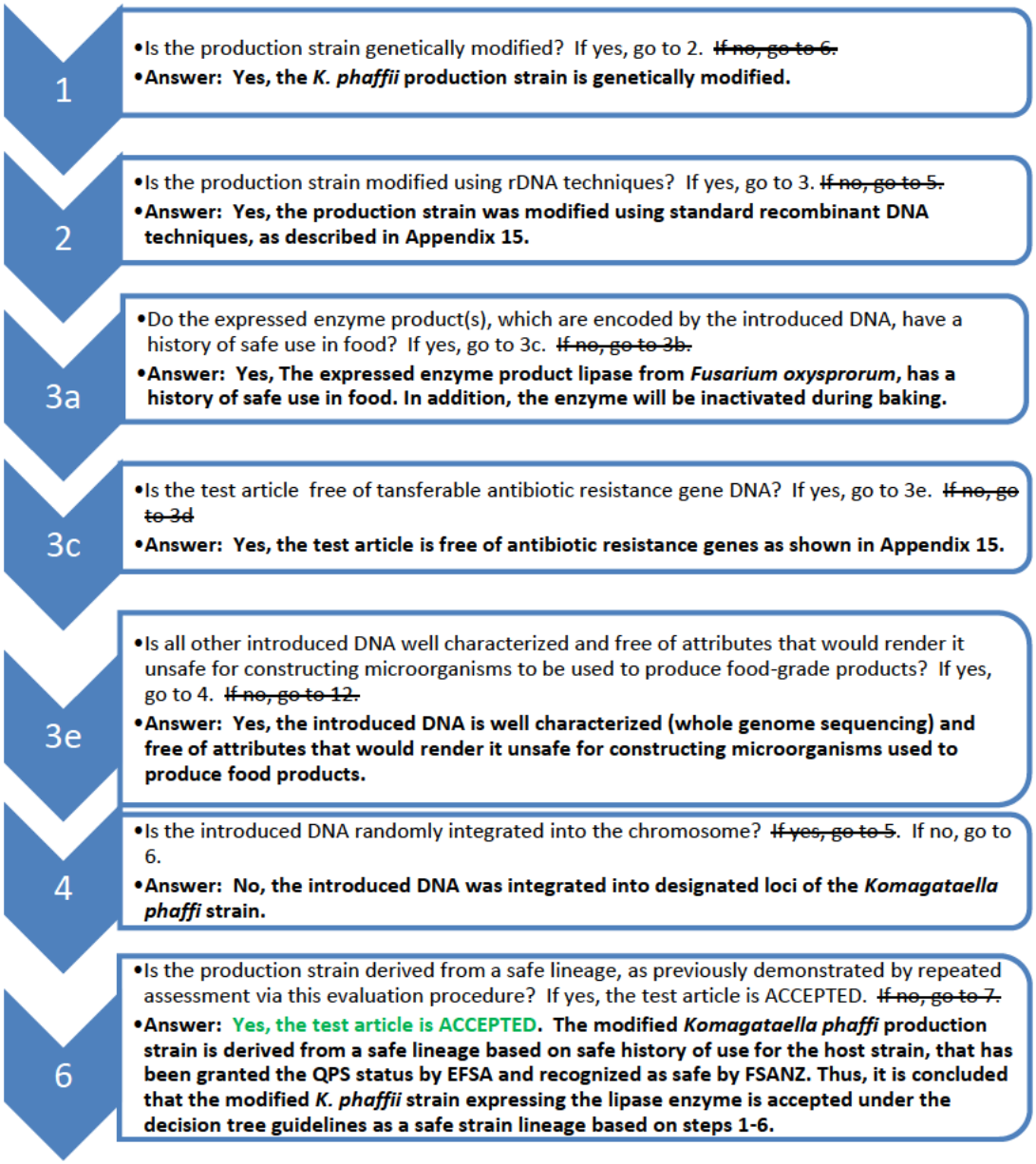

Figure 3: Pariza-Johnson Decision Tree Analysis of Lipase Enzyme Production Strain LALL-LI2

D.2 Information on the pathogenicity and toxicity of the source microorganism

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.

As highlighted in EFSA 'Scientific Guidance for the submission of dossiers on Food Enzymes' (section 1.1.10. Toxigenicity and pathogenicity), a production strain obtained by genetic modification of a host strain considered as safe can also be considered as safe as long as the genetic modification is well characterized, and no safety concerns arose from it.
The *K. phaffii* LALL-LI2 production strain is engineered from the safe host strain using homologous recombination with integration of DNA inserts containing copies of the synthetic native lipase gene from *F. oxysporum*. Indigenous vectors and other genetic material of *F. oxysporum* are not relevant because no material from the donor organism was used in the construction of the modified yeast strain. The lipase gene was stability integrated into the *K. phaffii* at predicted locations and are under the regulation of *K. phaffii* promoter and terminator. The insertions are limited in size, poorly mobilizable, well-characterized and do not encode or express any harmful substances. 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. Based on the EFSA approach, it can be considered that LALL-LI is a safe strain, neither toxigenic no pathogenic.

The toxigenicity and pathogenicity of *K. phaffii* was assessed through a (non-exhaustive) search of the publicly available scientific literature, and no reports were identified suggesting *K. phaffii* as a pathogenic potential or produces any toxigenic metabolites.

D.3 Information on the genetic stability of the source microorganism

Information regarding genetic stability of the source organism is provided in Appendix 15 - Source of the Food Enzyme (CONFIDENTIAL)

E Additional Information Related to the Safety of an Enzyme Processing Aid Derived from a Genetically-Modified Microorganism

E.1 Information on the methods used in the genetic modification of the source organism

All the requested information regarding the genetic modification of the source organism is provided in Appendix 15 - Source of the Food Enzyme (CONFIDENTIAL).

F Information Related to the Dietary Exposure of the Processing Aid

F.1 A list of food or food groups likely to contain the processing aid or its metabolites

Based on the food group descriptions in the Food Additives Schedule 15 (table S15-5), lipase enzyme from a *Komagataella phaffii* LALL-LI2 would be used on the following food groups:

- **7 Bread and bakery products**

F.2 The levels of residues of the processing aid or its metabolites for each food or food group

The lipase food enzyme from LALL-LI2 is proposed for use at the minimum levels required to achieve the desired technical effect in accordance with GMP.

The typical level of use for the food enzyme processing aid is estimated to go from 70 LBLU/kg flour to 2,000 LBLU/kg flour. Dosages used may vary depending on the type of baking product, the baking process and recipe and the desired effect level.
As per the data available in Appendix 7 - Certificates of Analysis, the average lipase activity of the lipase enzyme for the 3 batches submitted is 18.3 LBLU/mg of enzyme preparation, corresponding to 367.3 LBLU/mg TOS.
Therefore, the typical maximum level of use for the food enzyme is 2,000 LBLU/kg flour/ 18.3 LBLU/mg = 109.3 mg of lipase enzyme preparation / kg flour, corresponding to 2,000 LBLU/kg flour/ 367.3 LBLU/mg TOS = 5.4 mg TOS/ kg flour.

Application	Raw Material (RM)	Maximal recommended use levels (mg TOS[(1)]/kg RM)
Baking	Flour	5.4

[(1)] TOS (Total organic solids) = 100% - (A+W+D)
where A = % ash, W = % water, and D = % diluents and/or other formulation ingredients

The enzyme processing aid is added to the ingredients during the preparation of the dough, performs its technological function during dough handling, and is then denatured by heat during the baking step. In fact, as shown in Figure 4 below, the enzyme is completely deactivated after 15 min above 60°C.

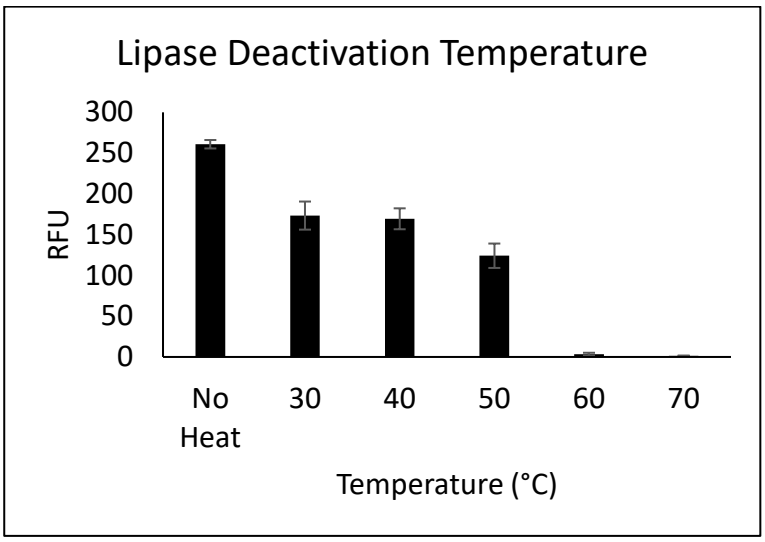

Figure 4: Thermal deactivation for the lipase from LALL-LI2

The lipase sample was prepared by diluting an aliquot from lot B76Z6Y. Reactions were prepared and incubated at temperatures ranging from 25°C (no heat) to 70°C for 15 minutes. Following the pre-incubation, 50 µL of the incubated samples was mixed with 50 µL DGGR lipase substrate (1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin ester, CAS Number: 195833-46-6) and incubated at 30°C for 10 minutes [1]. Reactions were stopped through addition of 50 µL 1 M sodium carbonate buffer and relative fluorescence was measured (Ex/Em 529 nm/ 600 nm). The values plotted in Figure 4 were normalized by subtracting the $RFU_{529/600}$ value of the blank sample.

[1]) During incubation of the enzyme and the DGGR substrate, enzymatic hydrolysis of the substrate produces a red-purple chromogenic compound, methylresorufin. This compound is also fluorogenic. Reactions can be stopped by addition of sodium carbonate buffer and fluorescence measured using an excitation wavelength (Ex) of 529 nm and an emission wavelength (Em) of 600 nm. The intensity of the fluorescence is proportional to the lipase enzymatic activity.

Dietary Exposure Assessment

The Budget Method was used to obtain an estimate of the potential dietary exposure to the lipase enzyme processing aid in foods intended for consumption for the general population on the basis that the lipase is used bread and bakery products as described above in Section F.1. 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 is specific to solid food. The result is an estimate of the dietary exposure to the food enzyme 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 assumed to be present in solid foods is based on the maximum level of the food enzyme in solid foods (*i.e.*, 5.4 mg TOS/kg flour, see above). This conservative approach is made

assuming that bread and bakery 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)[13]). This assumes that a typical adult weighing 70 kg consumes 0.88 kg of solid food which are produced using the food enzyme.

Theoretical Maximum Daily Intake of Enzyme

Based on conservative estimates of exposure calculated using the budget method, the TMDI of the lipase food enzyme from all solid foods was calculated to be 0.068 mg TOS/kg body weight/day. The calculations for the derivation of the TMDI of the food enzyme from all solid foods and the resulting total estimated intakes are presented in Table 5.

Table 5: TMDI of Lipase Based on the Maximum Use Levels in Solid Foods Using the Budget Method

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	5.4	**0.068**

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 lipase food enzyme is intended to be used as a processing aid in replacement for currently approved lipases and will not increase the potential consumer exposure to lipase. The estimated human exposure to the lipase food enzyme was calculated using the Budget Method, reflecting the proposed uses of the enzyme. The assumptions have been conservative to ensure there is no under-estimation of intakes of the food enzyme. The Budget Method uses standard values to calculate the TDMI to the food enzyme based on conservative assumptions regarding dietary intake of solid foods. In the assessment, the enzyme was

[13] 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.

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 0.068 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.

Since no toxicological assessment is considered necessary by Danstar Ferment AG, no margin of exposure has been calculated.

F.3 For foods or food groups not currently listed in the most recent Australian or New Zealand National Nutrition Surveys (NNSs), information on the likely level of consumption

Not applicable.

F.4 The percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid

Based on the conservative approach applied to calculate the dietary exposure to the enzyme processing aid using the Budget Method (see section F.2 above), it is assumed that all bakery products are produced using the lipase enzyme as a processing aid at the maximum recommended dose.

F.5 Information relating to the levels of residues in foods in other countries

The Budget Method represents a worst-case scenario; Therefore, the same level of residues in foods as estimated in section F.2 is expected in other countries where the enzyme processing aid would be used.

F.6 For foods where consumption has changed in recent years, information on likely current food consumption

Not applicable.

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Appendices

Non-Confidential Appendices

The following non-confidential appendices are provided on the following pages.

Appendix 1	Statutory Declaration
Appendix 2	Checklists
Appendix 5	Manufacturing Process Flow Chart
Appendix 7	Certificates of Analysis
Appendix 8	Total Plate Count Method
Appendix 9	Coliforms Method
Appendix 10	Antimicrobial Activity Method
Appendix 12	Step-by-step Process for Toxin Search

Confidential Appendices

The following confidential appendices are provided in a separate document.

Appendix 3	Technological Effect of the Enzyme Processing Aid (CONFIDENTIAL)
Appendix 4	Lipase Activity Determination Method (CONFIDENTIAL)
Appendix 6	Manufacturing Process – List of Raw Materials and Processing Aids (CONFIDENTIAL)
Appendix 11	Determination of Absence of Production Strain (CONFIDENTIAL)
Appendix 13	Dynamic Upper GI Simulation Report Lipase from LALL-LI (CONFIDENTIAL)
Appendix 14	AllergenOnline Search (CONFIDENTIAL)
Appendix 15	Source of the Food Enzyme (CONFIDENTIAL)
Appendix 16	Whole Genome Sequencing Analysis Report (CONFIDENTIAL)

Appendix 1 - Statutory Declaration

A statutory declaration according to the information provided in 'Making a Commonwealth statutory declaration with a witness' and 'Making a Commonwealth statutory declaration overseas' will be provided when the application is formally submitted.

Appendix 2 - Checklists

	Mandatory Requirements	Check	Page Number	Comments
General requirements for applications (3.1.1)	A. Form of the application			
	Application in English	☒	NA [1]	
	Executive summary	☒	NA	Submitted separately
	Relevant sections of Part 3 clearly identified	☒	NA	Sections are identified throughout the application
	Pages sequentially numbered	☒	NA	
	Electronic copy (searchable)	☒	NA	
	All references provided	☒	NA	
	B. Applicant details	☒	5	
	C. Purpose of the application	☒	5	
	D. Justification for the application			
	D.1.1 Costs and benefits of the application	☒	6	
	D.1.2 Impact on international trade	☒	6	
	E Information to support the application			
	E.1 Data requirements	☒	7	
	F. Assessment procedure	☒	7	
	G. Confidential commercial information (CCI)	☒	7	
	H. Other confidential information	☒	8	
	I. Exclusive capturable commercial benefit (ECCB)	☒	8	
	J. International and other national standards			
	J.1 International Standards	☒	8	
	J.2 Other national standards or regulations	☒	9	
	K. Statutory declaration	☒	9	
	L. Checklist			
	3.1.1 Checklist	☒	9	
	All page number references from application included	☒	9	
	Any other relevant checklists for Chapters 3.2–3.7	☒	9	
Processing aids (3.3.2)	A.1 Information on the type of processing aid	☒	10	
	A.2 Information on the identity of the processing aid	☒	10	
	A.3 Information on the chemical and physical properties of the processing aid	☒	13	
	A.4 Manufacturing process	☒	15	
	A.5 Specification for identity and purity	☒	18	
	A.6 Analytical method for detection	NA	NA	Not applicable for enzymes used as processing aids

	B. Information Related to the Safety of a Chemical Processing Aid	NA	NA	Not applicable for enzymes processing aids
	C.1 General information on the use of the enzyme as a food processing aid in other countries	☒	20	
	C.2 Information on the potential toxicity of the enzyme processing aid	☒	21	
	C.3 Information on the potential allergenicity of the enzyme processing aid	☒	26	
	C.4 Safety assessment reports prepared by international agencies or other national government agencies, if available	☒	27	
	D.1 Information on the source microorganism	☒	28	
	D.2 Information on the pathogenicity and toxicity of the source microorganism	☒	30	
	D.3 Information on the genetic stability of the source organism	☒	30	
	E.1 Information on the methods used in the genetic modification of the source organism	☒	31	
	F.1. A list of foods or food groups likely to contain the processing aid or its metabolites	☒	31	
	F.2 The levels of residues of the processing aid or its metabolites for each food or food group	☒	31	
	F.3 For foods or food groups not currently listed in the most recent Australian or New Zealand National Nutrition Surveys (NNSs), information on the likely level of consumption	☒	34	
	F.4 The percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid	☒	34	
	F.5 Information relating to the levels of residues in foods in other countries	☒	34	
	F.6 For foods where consumption has changed in recent years, information on likely current food consumption	☒	34	

(1) NA: Not Applicable

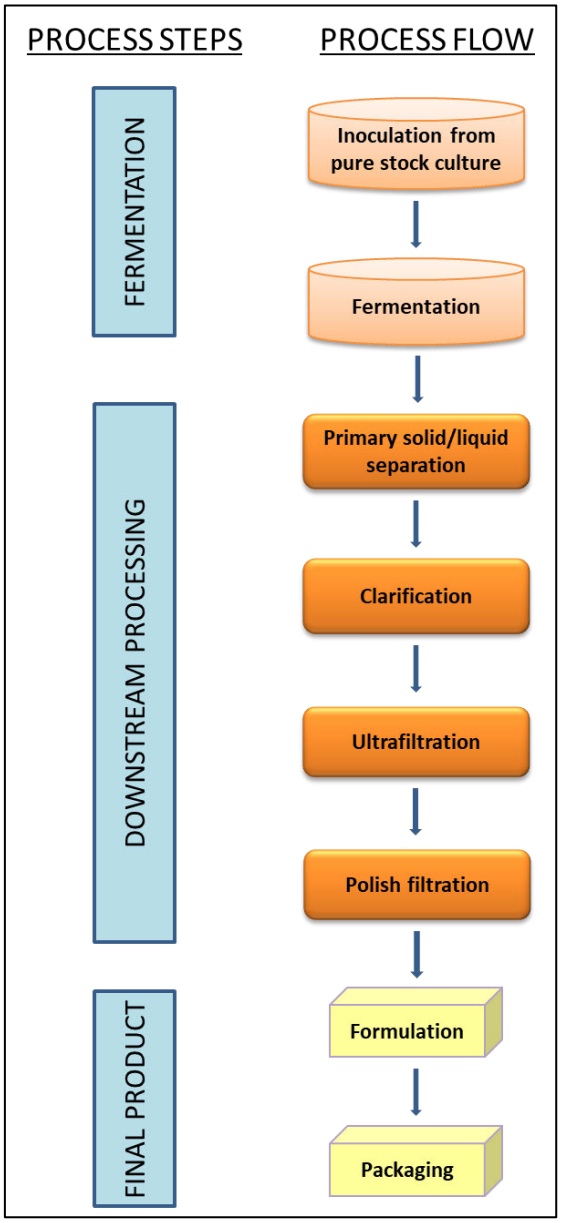
Appendix 3 - Technological effect of the Enzyme Processing Aid (CONFIDENTIAL)

Appendix 3 is provided in a separate document.

Appendix 4 - Lipase Activity Determination Method (CONFIDENTIAL)

Appendix 4 is provided in a separate document.

Appendix 5 - Manufacturing Process Flow Chart



Appendix 6 - Manufacturing Process – List of Raw Materials and Processing Aids (CONFIDENTIAL)

Appendix 6 is provided in a separate document.

Appendix 7 - Certificates of Analysis

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Certificate of analysis

Principal enzyme activity: Lipase
IUBMB number: EC 3.1.1.3
Production organism: *Komagataella phaffii* LALL-LI2
Lot Number: HH783P

Parameter	Specifications	Method	Results
Enzyme activity			
Lipase (LBLU/g)	> 10,000	Internal method	12,914
Microbiological parameters			
Total plate count (CFU/g)	≤ 50,000	Internal method	< 100
Coliforms (CFU/g)	≤ 30	Internal method	< 10
E. coli (/25g)	Not detected	ISO 16649-3	Not detected
Salmonella (/25g)	Not detected	NEOGEN® ANSR kit for Salmonella (AOAC certified)	Not detected
Antibacterial activity	Absent	Internal method	Absent
Production organism (/g)	Absent	Internal method	Absent
Heavy metals			
Lead (ppm)	≤ 5	AOAC 2011.14	0.028
Arsenic (ppm)	≤ 1	AOAC 2011.14	< 0.02
Cadmium (ppm)	≤ 0.5	AOAC 2011.14	< 0.008
Mercury (ppm)	≤ 0.5	AOAC 2011.14	< 0.01

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Certificate of analysis

Principal enzyme activity: Lipase
IUBMB number: EC 3.1.1.3
Production organism: *Komagataella phaffii* LALL-LI2
Lot Number: B76Z6Y

Parameter	Specifications	Method	Results
Enzyme activity			
Lipase activity (LBLU/g)	> 10,000	Internal method	16,351
Microbiological parameters			
Total plate count (CFU/g)	≤ 50,000	Internal method	< 100
Coliforms (CFU/g)	≤ 30	Internal method	< 10
E. coli (/25g)	Not detected	ISO 16649-3	Not detected
Salmonella (/25g)	Not detected	NEOGEN® ANSR kit for Salmonella (AOAC certified)	Not detected
Antibacterial activity	Absent	Internal method	Absent
Production organism (/g)	Absent	Internal method	Absent
Heavy metals			
Lead (ppm)	≤ 5	AOAC 2011.14	0.047
Arsenic (ppm)	≤ 1	AOAC 2011.14	< 0.02
Cadmium (ppm)	≤ 0.5	AOAC 2011.14	< 0.008
Mercury (ppm)	≤ 0.5	AOAC 2011.14	< 0.01

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Certificate of analysis

Principal enzyme activity:	Lipase
IUBMB number:	EC 3.1.1.3
Production organism:	*Komagataella phaffii* LALL-LI2
Lot Number:	BHC923

Parameter	Specifications	Method	Results
Enzyme activity			
Lipase activity (LBLU/g)	> 10,000	Internal method	25,621
Microbiological parameters			
Total plate count (CFU/g)	≤ 50,000	Internal method	< 100
Coliforms (CFU/g)	≤ 30	Internal method	< 10
E. coli (/25g)	Not Detected	ISO 16649-3	Not detected
Salmonella (/25g)	Not Detected	NEOGEN® ANSR kit for Salmonella (AOAC certified)	Not detected
Antibacterial activity	Absent	Internal method	Absent
Production organism (/g)	Absent	Internal method	Absent
Heavy metals			
Lead (ppm)	≤ 5	AOAC 2011.14	0.025
Arsenic (ppm)	≤ 1	AOAC 2011.14	< 0.02
Cadmium (ppm)	≤ 0.5	AOAC 2011.14	< 0.008
Mercury (ppm)	≤ 0.5	AOAC 2011.14	< 0.01

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Appendix 8 - Total Plate Count Method

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Determination of Total Plate Count in Food Enzymes

Objective

This test is used to measure the degree of contamination in food enzymes.

Material

- ✓ TPC agar
- ✓ Microwave
- ✓ Scale and metal spatula
- ✓ Bunsen burner and igniter
- ✓ 9 ml screw-on tubes of sterile tap water
- ✓ 90 ml screw-on bottle of sterile tap water
- ✓ Sterile petri dishes
- ✓ 1000 µL micropipettes and sterile filtered tips
- ✓ Vortex
- ✓ Cycloheximide solution 0.4%
- ✓ Incubator at 30°C
- ✓ Water bath at 37°C
- ✓ Water bath at 50°C

Method

1. Melt the TPC agar in the microwave. Let it cool in the bath at 50°C before using.
2. Aseptically weigh 10.0 g of the sample into a dilution bottle containing 90 ml of sterile tap H_2O. This represents the 10^{-1} dilution. Vortex well.
3. For enzyme powders, hydrate in a water bath at 37°C for 10 minutes without stirring. Afterwards, mix well and return to the water bath for 10 minutes with 130 rpm stirring or stir manually every 2 minutes. This step is not necessary for liquid enzyme samples.
4. From tube 10^{-1}, proceed by serial dilution until the appropriate dilutions are obtained. Add 1 mL of the desired dilution per petri. Perform in duplicate. (NB: vortex well between each dilution)
5. At the time of inoculation, add sterile 0.4% cycloheximide to the TPC medium tempered at 50°C. To limit the risks of contamination, the cycloheximide is separated beforehand in sterile 3.2 ml conical tubes for 800 mL of medium.
6. Pour 10 to 15 ml of medium into the inoculated petri dish. Distribute delicately with movements of figure-eight.
7. Once the petri dishes have solidified, incubate the inverted petri dishes at 30°C for 48 hours.
8. Count bacterial colonies after 48 hours. Apply the appropriate correction factor for the dilution. The result is expressed in number of colonies/g of sample.

1/1

Appendix 9 - Coliforms Method

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Determination of Total Coliform Count with Harlequin ™ HAL008 (chromogenic medium)

Objective

This test is used to measure the degree of total coliform contamination in food enzymes.

Material

- ✓ Harlequin medium in 100mm plates (house cast)
- ✓ Scale and metal spatula
- ✓ Bunsen burner and igniter
- ✓ 9 ml screw-on tubes of sterile tap water
- ✓ 1000 µL micropipettes and sterile filtered tips
- ✓ Vortex
- ✓ Incubator at 37°C
- ✓ Water bath at 37°C

Method

1. Remove the plates of Harlequin chromogenic medium from the fridge so that they are at room temperature (about 15 minutes).
2. Aseptically weigh 10.0 g of the liquid enzyme or the enzyme powder into a dilution bottle containing 90 mL of sterile tap water. This represents the 10^{-1} dilution. Vortex well.
3. For the powder samples, hydrate them in a bath at 37°C for 10 minutes without shaking then return to a shaking bath for 10 minutes at 120 rpm or stir manually every 2 minutes. This step is not necessary for liquid samples.
4. Place 1 mL of this dilution on a plate of Harlequin medium and disperse well on the plate.
5. Once the liquid is well absorbed on the plate (between 15 and 30 minutes depending on the consistency), incubate the inverted petri at 37°C for 24 hours.
6. Count the bacterial colonies after 24 hours of incubation. Apply the appropriate correction factor based upon the dilution. The result is expressed in colonies of total coliforms (coliforms + *E. coli*)/1 g of sample.

1/1

Appendix 10 - Antibacterial Activity Method

Determination of Antibacterial Activity in Food Enzymes

Objective

This procedure is to determine antibacterial activity in food enzymes following JECFA guidelines[1].

Material and Methods

1. Enzyme sample preparation

Enzyme samples are mixed with sterile DI water to a final concentration of 10% (w/v for powder enzyme samples and v/v for liquid enzyme samples). 0.1 mL of the enzyme preparation is applied to a sterile (autoclaved) filter disk (Whatman® Antibiotic Assay Discs, 9 mm) to saturate the disk with the enzyme preparation. Six discs of are prepared for each sample to test on six bacterial strains.

2. Bacterial culture preparation

Six strains were acquired as lyophilized preparations from ATCC (https://www.atcc.org/en.aspx): *Staphylococcus aureus* (ATCC 6538); *Escherichia coli* (ATCC 11229); *Bacillus cereus* (ATCC 11778); *Bacillus circulans* (ATCC 4516); *Streptococcus pyogenes* (ATCC 12344); and *Serratia marcescens* (ATCC 14041),and revived following manufacturer's instructions. These strains have been cryopreserved in TSB (Tryptic Soy Broth) with 20% glycerol at -80°C. The table below lists the internal designations for these strains and their identity:

M26869	ATCC 6538	*Staphylococcus aureus subsp. aureus*
M26870	ATCC 12344	*Streptococcus pyogenes* Strain Type 1
M26871	ATCC 11229	*Escherichia coli*
M26872	ATCC 11778	*Bacillus cereus*
M26873	ATCC 4516	*Bacillus circulans*
M26874	ATCC 14041	*Serratia marcescens*

Cultures for this experiment set are inoculated directly from the glycerol stocks of these strains in Tryptic Soy Broth. The cells are let grown for 24 hours with shaking at 220 rpm and 37°C. Tryptic Soy Agar (TSA) plates are prepared using sterile technique as follows:

Step 1: 15 mL TSA is poured into each Petri dish and allowed to solidify.

Step 2: A second 10 mL Bacterially Seeded TSA layer will be applied to each plate. Seeded TSA is prepared by diluting the 24 hour bacterial cultures 1:10 into warm TSA (45-50°C) (1:20 dilution for *Streptococcus pyogenes*), and overlaying the 15 mL-TSA plates with 10 mL of the seeded TSA.

3. Antibacterial activity testing

The prepared discs are placed on the surface of the hardened culture plates. Plates are incubated at 4°C for overnight to allow proper diffusion. Following the 4°C incubation, plates are incubated at 37°C for 24 hours. After incubation, plates are visually examined for the presence of a clear zone around the disk. If a clear zone of a total diameter of 12 mm is observed, the enzyme preparation is considered inhibitory for this strain.

As a positive control, Tetracycline Antimicrobial Susceptibility discs can be used, one per plate (ThermoFisher CT0054B).

References

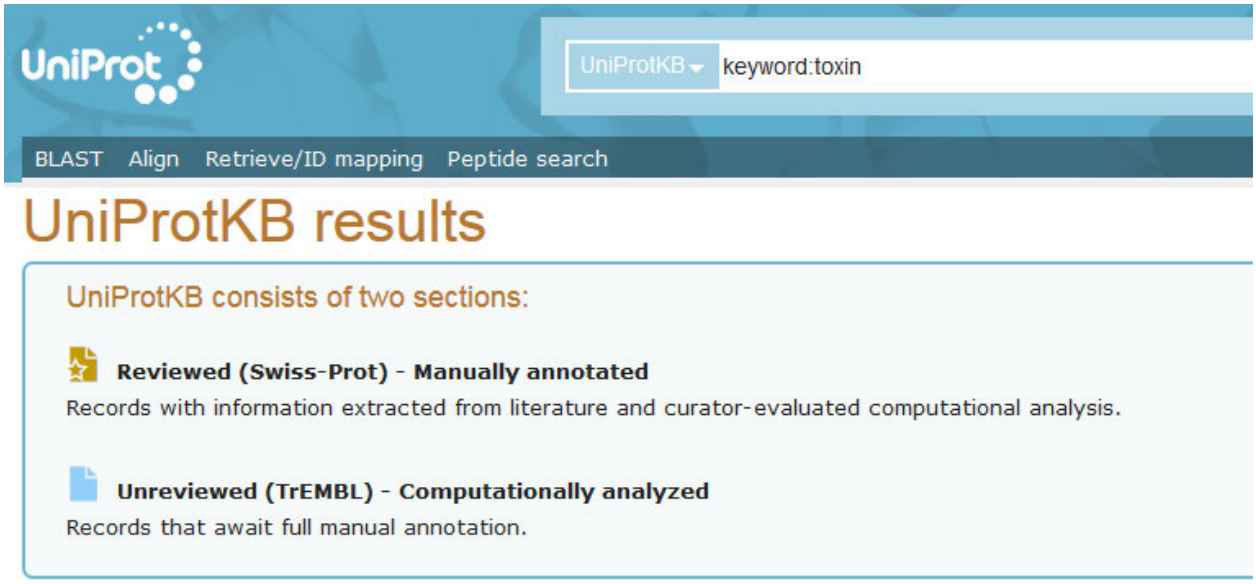
1. Joint FAO/WHO Expert Committee on Food Additive. COMBINED COMPENDIUM OF FOOD ADDITIVE SPECIFICATIONS, volume 4 (Analytical methods, test procedures and laboratory solutions used by and referenced in the food additive specifications). http://www.fao.org/3/a0691e/a0691e.pdf

Appendix 11 - Determination of absence of production strain (CONFIDENTIAL)

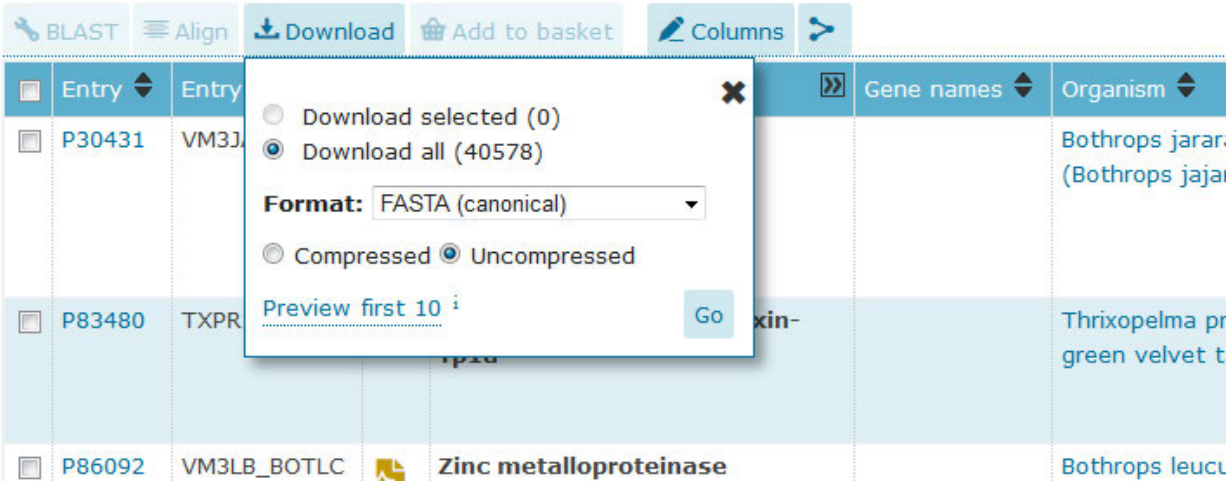
Appendix 11 is provided in a separate document.

Appendix 12 - Step-by-step process for toxin search

1. In Uniprot (https://www.uniprot.org/) search UniProtKB with the terms "keyword:toxin". You will get results from Swiss-Prot and TrEMBL databases.

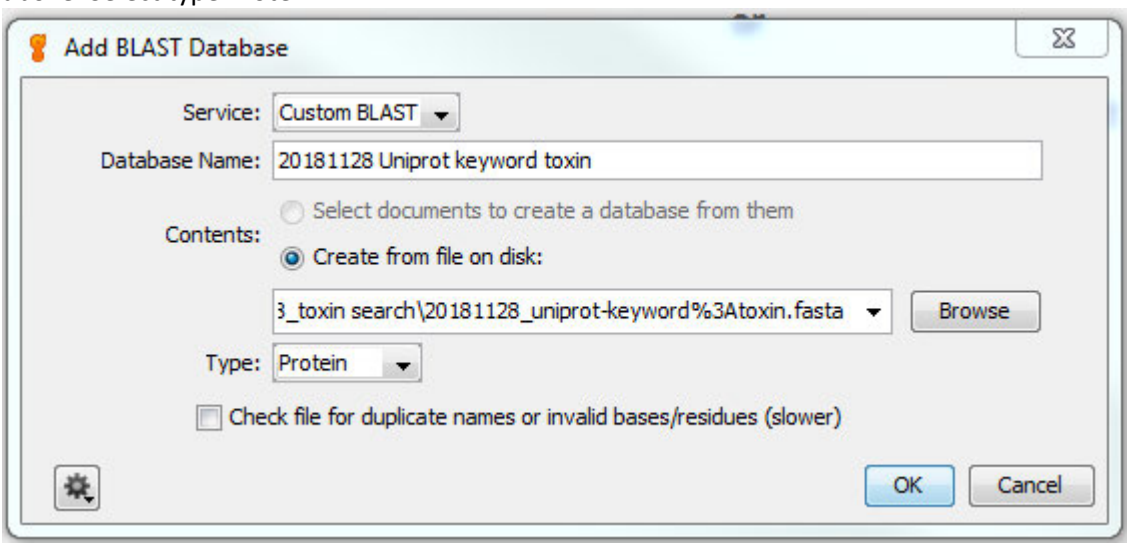


2. Download the resulting list as an uncompressed file. Save the file in a location where you can easily find it. It might help to note the data of download in the file name so that it's easy to remember when the list was downloaded (this is important because the Uniprot toxin database might change over time). In any case you will want to note the date of download for your search writeup.

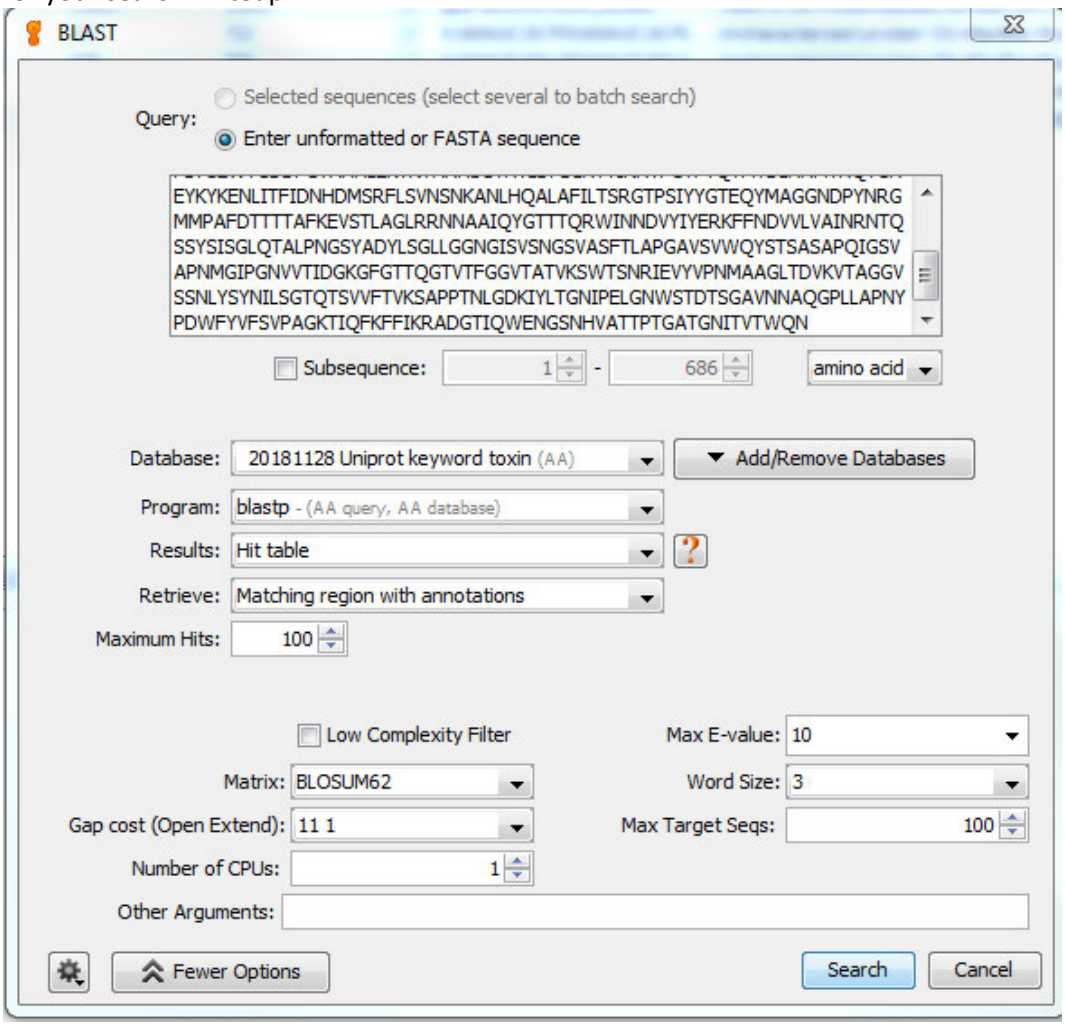


3. Open Geneious and make sure you have the Custom BLAST service: Tools→Add/Remove Databases→Set up BLAST Services.

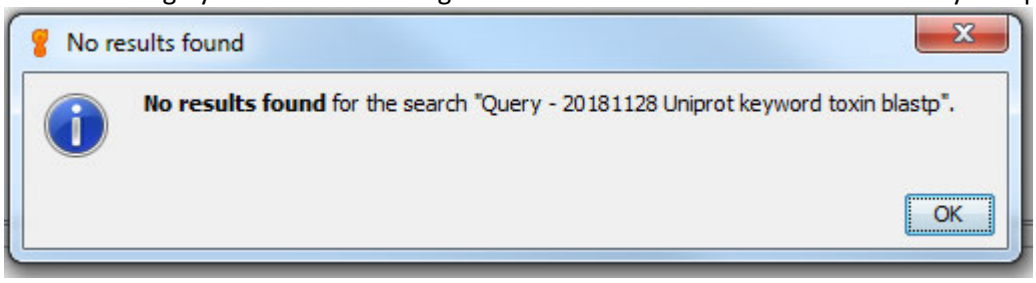
4. Make a custom database in Geneious with Tools→Add/Remove Databases→Add Sequence Database. Select service Custom BLAST, give the database a name, and create it from your saved .fasta file from step 2 above. Select type Protein.



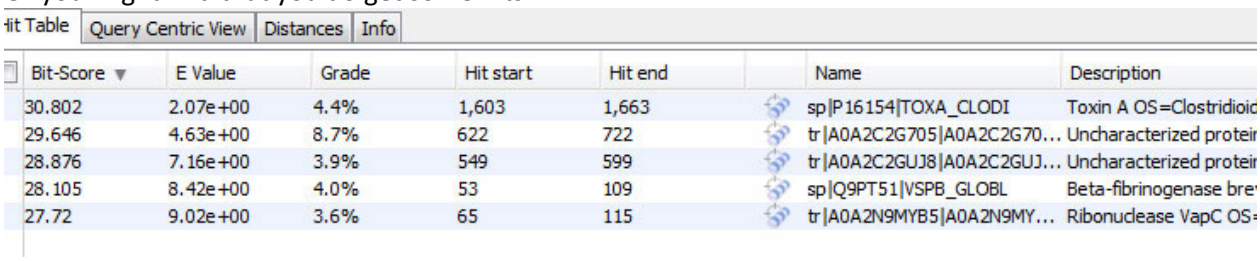
5. Perform the BLAST search in Geneious by clicking BLAST in the top bar. In the window that pops up, enter the protein sequence of interest, the custom database to search against, blastp for program, and make any adjustments or filters desired. For example, you could set the max E-value for a "hit" to a certain level (if you do this, you might want to run the BLAST with a few different E-value levels). Note the search settings for your search writeup.



6. Look through your results! You might find that there are no results found for your specific query:

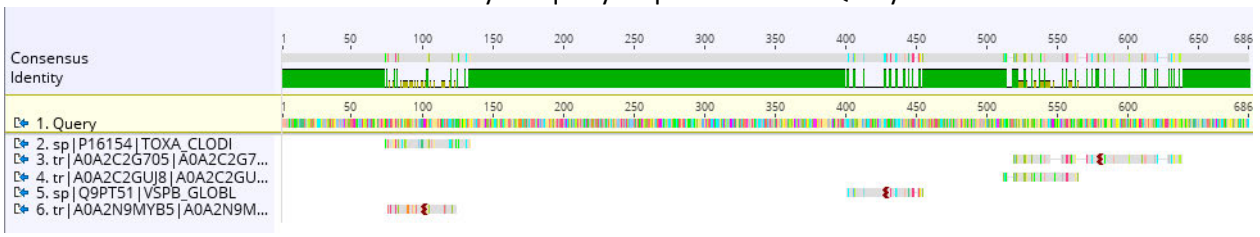


Or you might find that you do get some hits:



Bit-Score	E Value	Grade	Hit start	Hit end	Name	Description
30.802	2.07e+00	4.4%	1,603	1,663	sp|P16154|TOXA_CLODI	Toxin A OS=Clostridioid
29.646	4.63e+00	8.7%	622	722	tr|A0A2C2G705|A0A2C2G70...	Uncharacterized protein
28.876	7.16e+00	3.9%	549	599	tr|A0A2C2GUJ8|A0A2C2GUJ...	Uncharacterized protein
28.105	8.42e+00	4.0%	53	109	sp|Q9PT51|VSPB_GLOBL	Beta-fibrinogenase bre
27.72	9.02e+00	3.6%	65	115	tr|A0A2N9MYB5|A0A2N9MY...	Ribonuclease VapC OS=

You can look at the how the hits match your query sequence in the "Query Centric View" tab:



7. Write up your results! Include the date you searched the Uniprot database, the BLAST settings you used, etc.

Appendix 13 - Dynamic Upper GI Simulation Report Lipase from LALL-LI (CONFIDENTIAL)

Appendix 13 is provided in a separate document.

Appendix 14 - AllergenOnline Search (CONFIDENTIAL)

Appendix 14 is provided in a separate document.

Appendix 15 - Source of the Food Enzyme (CONFIDENTIAL)

Appendix 15 is provided in a separate document.

Appendix 16 - Whole Genome Sequencing Analysis Report (CONFIDENTIAL)

Appendix 16 is provided in a separate document.

Appendix 17 - FastQC Reports (CONFIDENTIAL)

Appendix 17 is provided in a separate document.

Annexes

The following annex is provided separately:
Annex 1 - Uniprot_db_keyword-toxin_2023-05-17