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Lipase from *Komagataella phaffii*

An application to amend the Australia New Zealand Food Standards Code with a lipase preparation produced by a genetically modified strain of *Komagataella phaffii*

Chr. Hansen Pty Ltd
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Executive summary

The present application seeks to amend Schedule 18—Processing aids of the Australia New Zealand Food Standards Code (the Code) to approve a triacylglycerol lipase enzyme preparation produced by Chr. Hansen.

Proposed change to Australia New Zealand Food Standards Code – Schedule 18—Processing aids

Schedule 18—Processing aids is proposed to be amended to include a genetically modified strain of *Komagataella phaffii* expressing a triacylglycerol lipase from *Yarrowia lipolytica* as a permitted source for triacylglycerol lipase.

The application is applied for assessment by the general procedure.

Description of enzyme preparation

The enzyme is a triacylglycerol lipase (EC 3.1.1.3), commonly known as lipase.

The lipase catalyses the hydrolysis of triglycerides, diglycerides and monoglycerides to yield free fatty acids and monoglycerides, diglycerides and glycerol.

The enzyme is produced by submerged fermentation of a *Komagataella phaffii* microorganism expressing a lipase from *Yarrowia lipolytica*.

The lipase enzyme preparation is available as a liquid preparation complying with the JECFA recommended purity specifications for food-grade enzymes.

The producing microorganism, *Komagataella phaffii*, is absent from the commercial enzyme product.

Use of the enzyme

The lipase enzyme preparation is used as a processing aid in cheese production, production of flavouring preparations from dairy products (enzyme-modified dairy ingredients), and production of plant-based analogues of milk and milk products. The lipase catalyses the hydrolysis of triglycerides, diglycerides and monoglycerides to yield free fatty acids and monoglycerides, diglycerides and glycerol.

Benefits

The benefit of using the lipase in cheese production is:

- Helping develop the flavour of specific cheeses

The benefit of using lipase in the production of flavouring preparations from dairy products is:

- To develop the characteristic flavour

The benefits of using lipase in the production of production of plant-based analogues of milk and milk products are:

- Helping plant-based products develop a dairy-like flavour.
- Helping plant-based products achieve a desirable texture while limiting the addition of saturated or hydrogenated fat.

Safety evaluation

The safety of the production organism and the enzyme product has been thoroughly assessed:

- The production organism has a long history of safe use as a production strain in the food industry and is known not to produce any toxic metabolites.
- The genetic modifications in the production organism are well-characterised and safe and the recombinant DNA is stably integrated into the production organism and unlikely to pose a safety concern.
- The enzyme preparation complies with international specifications ensuring the absence of contamination by toxic substances or noxious microorganisms.
- Sequence homology assessment to known allergens and toxins shows that oral intake of the lipase is unlikely to pose food allergenic or toxic concern.

Furthermore, the safety of the lipase preparation was confirmed by external expert groups, as follows:

- Denmark: The enzyme preparation was safety assessed resulting in the authorisation of the enzyme product by the Danish Veterinary and Food Administration.

Conclusion

Based on the safety evaluation, confirmed by the above-mentioned bodies, we respectfully request the inclusion of the lipase in Schedule 18—Processing aids.

Introduction

The present application describes a lipase enzyme preparation produced by submerged fermentation of a *Komagataella phaffii* microorganism producing a lipase from *Yarrowia lipolytica*.

The enzyme is a triacylglycerol lipase (EC 3.1.1.3), commonly known as lipase. The enzyme catalyses the hydrolysis of triglycerides, diglycerides and monoglycerides to yield free fatty acids and monoglycerides, diglycerides and glycerol.

The enzyme in scope of this application is intended to be used in cheese production, the production of flavouring preparations from dairy products (enzyme-modified dairy ingredients), and the production of plant-based analogues of milk and milk products.

The following sections describe in detail the construction of the genetically modified *Komagataella phaffii* used as the production organism, the production process, the product specification, the application of the enzyme preparation and finally the safety evaluation of the product, which has been carried out confirming the safety of the product for its intended use.

The documentation has been elaborated according to the Application Handbook from Food Standards Australia New Zealand as of 1 July 2019, applied as relevant for an enzyme application, *i.e.* outlining the following section:

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

NB! In Appendix 2.1, the lipase enzyme preparation is described by its commercial name, SpiceIT M100.

Chapter 3.1, General requirements for applications

A Executive summary

An Executive Summary is provided as a separate copy together with this application.

B Applicant details

(a) **Applicant's name/s**

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(f) **Nature of applicant's business**
Biotechnology

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

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C Purpose of the application

This application is submitted to provide for amendment of the Australia New Zealand Food Standards Code, Schedule 18—Processing aids to include a genetically modified strain of *Komagataella phaffii* expressing a triacylglycerol lipase from *Yarrowia lipolytica* as a permitted source for triacylglycerol lipase.

D Justification for the application

The need for the proposed change

Schedule 18—Processing aids contains a list of permitted enzymes of microbial origin, among others triacylglycerol lipase (EC 3.1.1.3) from different microbial sources. Schedule 18—processing aids does not contain a triacylglycerol lipase (EC 3.1.1.3) from *Komagataella phaffii* (formerly known as *Pichia pastoris*) containing the gene for lipase from *Yarrowia lipolytica* although *P. pastoris* are listed for UDP-glucosyltransferases.

K. phaffii is one of the most widely used yeast species for the expression of heterologous proteins, and it has a long history of safe use as a production organism in the food industry (J. L. Cereghino & Cregg, 2000; EFSA Scientific Committee, 2007). The species was previously known as *P. pastoris*. In 1995, it was proposed that *P. pastoris* should be classified as a distinct genus called *Komagataella* (Yamada et al., 1995). Subsequently, based on further phylogenetic analysis, the *P. pastoris* strains most widely used in research and industrial production of recombinant proteins were re-classified as *Komagataella pastoris* and *K. phaffii* (Kurtzman, 2005, 2009).

P. pastoris was initially included in EFSA's list of microorganisms with Qualified Presumption of Safety (QPS) status in 2007 (EFSA Scientific Committee, 2007). In the 2010 update, it was reclassified as *K. pastoris* (EFSA BIOHAZ Panel, 2010), and later *K. phaffii* was recognized as a new taxonomic unit (EFSA BIOHAZ Panel, 2018ab, 2020). The QPS status of *K. phaffii* (and *K. pastoris*) is still maintained in the current list, with the condition that it is only used for 'production purposes only', implying the absence of viable cells of the production organism in the final product (EFSA BIOHAZ Panel, 2023a, 2023b). Furthermore, in Schedule 26 of the Australia New Zealand Food Standards Code, *K. phaffii* (formerly known as *P. pastoris*) is an approved source for the production of soy leghemoglobin.

The advantages of the proposed change over the status quo

The lipase preparation is used as a processing aid during the manufacture of lipid-based products. The lipase catalyses the hydrolysis of triglycerides, diglycerides and monoglycerides to yield free fatty acids and monoglycerides, diglycerides and glycerol.

The benefit of using the lipase in cheese production is:

- Helping develop the flavour of specific cheeses

The benefit of using lipase in the production of flavouring preparations from dairy products is:

- To develop the characteristic flavour

The benefits of using lipase in the production of production of plant-based analogues of milk and milk products are:

- Helping plant-based products develop a dairy-like flavour.
- Helping plant-based products achieve a desirable texture while limiting the addition of saturated or hydrogenated fat.

D.1 Regulatory impact information

D.1.1 Costs and benefits of the application

The application is not likely to place costs or regulatory restrictions on industry or consumers. Inclusion of the lipase enzyme in Schedule 18—Processing aids will provide the food and beverage industry with the opportunity to improve food processing and the quality of the finished food. For the government, the burden is limited to necessary activities for a variation of Schedule 18—Processing aids.

D.1.2 Impact on international trade

The application is not likely to cause impact on international trade.

E Information to support the application

E.1 Data requirements

No public health and safety issues related to the proposed change are foreseen. As outlined in sections 3.3.2 C, D, E, F, the lipase is produced by submerged fermentation of a genetically modified *K. phaffii* strain.

The safety of the production organism and the enzyme product has been thoroughly assessed:

- The production organism has a long history of safe use as a production strain in the food industry and is known not to produce any toxic metabolites.
- The genetic modifications in the production organism are well-characterised and safe and the recombinant DNA is stably integrated into the production organism and unlikely to pose a safety concern.
- The enzyme preparation complies with international specifications ensuring absence of contamination by toxic substances or noxious microorganisms
- Sequence homology assessment to known allergens and toxins shows that oral intake of the lipase is unlikely to pose food allergenic or toxic concern.

F Assessment procedure

Because the application is for a new source organism for an existing enzyme in the Code, it is considered appropriate that the assessment procedure is characterised as “General Procedure, Level 1”.

G Confidential commercial information (CCI)

Detailed information on the raw materials used in production of the enzyme preparation and construction and characteristics of the genetically modified production strain are provided in **Appendix 4** and **6**, respectively. Summaries of the information are given in section A.4 and 3.3.2 E. The formal request for treatment of selected parts of **Appendix 4** and **6** as confidential commercial information (CCI) is included as **Appendix 1.1**.

H Other confidential information

Apart from the selected parts of **Appendix 4** and **6** identified as confidential commercial information (CCI), no other information is requested to be treated as confidential.

I Exclusive capturable commercial benefit (ECCB)

This application is not expected to confer an Exclusive Capturable Commercial Benefit.

J International and other national standards

J.1 International Standards

Use of enzymes as processing aids for food production is not restricted by any Codex Alimentarius Commission (Codex) Standards.

J.2 Other national standards or regulations

With few exceptions on national, commodity standards, use of enzymes as processing aids for food production is in general not restricted by standards or regulations in other countries.

K Statutory declaration

The Statutory Declaration is provided as a separate document together with this submission.

L Checklist

This application concerns an enzyme product intended to be used as a processing aid. Therefore, the relevant documentation according to the Application Handbook from Food Standards Australia New Zealand as of 1 July 2019, are the following sections:

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

Accordingly, the checklist for General requirements as well as the Processing aids part of the checklist for applications for substances added to food was used and is included as **Appendix 1.2** and **1.3**.

Chapter 3.3, Guidelines for applications for substances added to food

3.3.2 Processing Aids

The lipase enzyme preparation described in this application is representative of the commercial food enzyme product for which approval is sought.

A Technical information on the processing aid

A.1 Information on the type of processing aid

The lipase enzyme preparation belongs to the category of processing aids described in Schedule 18—Processing aids.

The lipase enzyme preparation is to be used in the food industry as a processing aid during the processing of raw materials containing lipids.

The lipase enzyme preparation is used in the following food manufacturing processes:

- cheese production,
- production of flavouring preparations from dairy products (enzyme-modified dairy ingredients), and
- production of plant-based analogues of milk and milk products

The highest dosage of the lipase is during cheese production, where dosages up to 100 LVU per litre of milk are used.

A.2 Information on the identity of the processing aid

A.2.1 Enzyme

Generic name	lipase
IUBMC nomenclature	triacylglycerol lipase
Enzyme classification (EC) number	EC 3.1.1.3
CAS number	9001-62-1

A.2.2 Enzyme preparation

The enzyme concentrate is formulated into a final enzyme preparation. The enzyme concentrate may be intended for a single enzyme preparation or a blend with other food enzymes and formulated as a liquid product depending on the characteristics of the intended food process in which it will be used.

The typical composition with an approximate percentage of the enzyme concentrate is:

Enzyme solids (TOS)[1]	1 %
Water	27%
Glycerol	15%
Sorbitol	57%

The method used to determine the LVU activity is enclosed in **Appendix 3**.

A.2.3 Host organism

The production strain for the lipase enzyme was developed from the *K.a phaffii* CBS 7435 (synonymously NRRL Y-11430) which was then deposited under DSM 34125. This isolate has been the focus of much research, and many of the commonly used heterologous expression systems are derived from this progenitor strain (J. L. Cereghino & Cregg, 2000). This includes lipase produced by *K. phaffii* DSM 34125, as well as all the food enzymes/ingredients produced with *K. phaffii* (*P. pastoris*) that have been concluded safe for their intended use in Australia/New Zealand, the United States, or the European Union. The taxonomic classification of the strain is:

Phylum	Ascomycota
Class	Saccharomycetes
Order	Saccharomycetales
Family	Phaffomycetaceae
Genus	*Komagataella*
Species	*Komagataella phaffii*

For a more detailed description of the host organism and the genetic modifications, please see section 3.3.2 E.

A.2.4 Donor organism

The donor for the lipase gene is *Yarrowia lipolytica*.

Phylum	Ascomycota
Class	Saccharomycetes
Order	Saccharomycetales
Family	Dipodascaceae
Genus	*Yarrowia*
Species	*Yarrowia lipolytica*

[1] TOS = Total Organic Solids, defined as: 100 % - water - ash - diluents

The lipase enzyme protein has not been protein-engineered. For a more detailed description of the donor and the donor gene, please see section 3.3.2 E and **Appendix 6**.

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

The enzyme is a triacylglycerol lipase (EC 3.1.1.3), commonly known as lipase. The lipase catalyses the hydrolysis of triglycerides, diglycerides and monoglycerides to yield free fatty acids and monoglycerides, diglycerides and glycerol.

The enzyme preparation is available as a liquid product. The appearance of the enzyme product is given in **Appendix 2.1**. The microorganisms used for fermentation will consume the majority of the raw materials. Subsequent downstream processes, such as filtration steps will remove remaining amounts of raw material. Thus, the final food enzyme product will not contain significant residual amounts of the nutrients used for fermentation. This is confirmed by regular spot-testing, where analytical measurements show no detectable amounts of the allergens listed in **Appendix 2.1**.

The lipase enzyme shows increasing activity from 0°C to its optimum at 35°C, with activity decreasing above this temperature. The enzyme's pH optimum is 4.5, with a broad activity range between pH 3-8, peaking at pH 4-5. Activity declines above pH 5. Furthermore, its thermostability profile shows the enzyme activity starts to decrease when exposed to temperatures above 35°C and becomes highly unstable when exposed to temperatures above 50°C.

No reaction products, which could not be considered normal constituents of the diet, are formed during the production or storage of the enzyme-treated food.

A.4 Manufacturing process

This section describes the processes used in manufacturing of the lipase enzyme product. Like all food enzymes, the food enzyme described in this dossier is manufactured in accordance with current Good Manufacturing Practice for Food (GMP) and the principles of Hazard Analysis of Critical Control Points (HACCP). The certificate proving the compliance of the production site with the FSSC 22000 standard is provided as supporting information, see **Appendix 4.1**.

A.4.1 Fermentation

The lipase is produced by submerged fed-batch pure culture fermentation of the genetically modified strain of *K. phaffii*, described in section 3.3.2 E.

A.4.1.1 Raw materials for fermentation

Materials used in the fermentation process (inoculum, seed and main fermentation) are:

- Potable water
- Carbon and nitrogen sources
- Salts and minerals
- Vitamins
- pH adjustment agents

- An antifoam

A list of the specific raw materials used during fermentation can be found in **Appendix 4.2**.

A.4.1.2 Inoculum

The production strain is stored as stock culture in small vials for the direct inoculation of the seed fermenter.

A.4.1.3 Seed fermentation

The inoculum is aseptically transferred to the seed fermenter containing sterilized fermentation medium. The seed fermentation is run under certain process conditions (i.e., temp and pH). The fermentation is run under controlled agitation and controlled air supply. When a sufficient amount of biomass has developed, the content of the seed fermenter is transferred to the main fermentation.

A.4.1.4 Main fermentation

Biosynthesis of the enzyme protein by the production micro-organisms occurs during the main fermentation. The fermentation in the main fermenter is operated as a fed-batch fermentation, whereby the content of the seed fermenter is aseptically transferred to the main fermenter containing fermentation medium. The main feed medium, which contains the raw materials as described in **Appendix 4.2**, including fermentation nutrients, trace elements and the antifoam is mixed and sterilised.

During the fed-batch process additional carbon source (sugar) and salts are added during the fermentation. To control the growth of the production micro-organism and the enzyme production, this medium (sugar and salts) is added based upon a fixed feeding profile. The main fermentation is run under the certain process conditions (i.e., temp, pH and dissolved oxygen concentration). The lipase and biomass concentrations in the fermentation broth are followed at regular intervals. Downstream processing (cross-flow micro-filtration) is initiated when the biomass content and enzyme titer in the process are within a feasible range.

In order to keep the cells active and avoid lysis, fermentation (including feeding) continues while the cross-flow filtration is running. Yeast cells remain in the bioreactor and the circulation loop while the triacylglycerol lipase passes the ceramic membrane via the permeate. To inactivate the production organism, the fermentation broth is heated. Samples are taken to document the complete inactivation of the microorganism.

A.4.2 Recovery

The purpose of the recovery process is:

- to separate the fermentation broth into biomass and product containing liquids

During fermentation the enzyme protein is largely excreted by the producing micro-organism into the fermentation medium. During recovery the enzyme-containing fermentation medium is separated from the biomass and purified by filtration.

A.4.2.1 Raw materials for recovery

A list of raw materials used for the recovery can be found in **Appendix 4.2**.

A.4.2.2 Micro-filtration

Crossflow filtration is a technique to separate the fermentation broth into retentate (the biomass-containing fraction) and permeate (the product-containing fraction). The permeate is collected for further recovery and formulation.

In the process, the method of tangential crossflow filtration with ceramic membrane modules is used. The product solution is pumped tangentially to the membrane at a suitable speed and the permeate is withdrawn transverse to the direction of flow. By selecting a membrane with a suitable separation size (cut-off and membrane surface) the yeast cells retain in the retentate (concentrate) and smaller particles and molecules (product) pass into the permeate. The permeate flow and thus the speed of the process are determined by the selected transmembrane pressure.

In order to achieve a high product concentration micro-filtration consist of two phases: a first phase extracting permeate to a critical/maximum biomass concentration without affecting the permeate flow to reduce volume and a second phase adding water while extracting permeate until obtaining a suitably low triacylglycerol lipase concentration in the retentate. The permeate is collected in a vessel for further downstream processing.

A.4.2.3 Polish filtration

The liquid (permeate) could still contain small insoluble particles (from medium composition) or unwanted molecules. Removal and/or reduction of these substances is done by a polish filtration step. For this process a specific depth filter is used, with a small pore size to remove smaller particles and ion-exchange characteristics to bind molecules with an isoelectric point (pI-value) lower than the product. The permeate is pumped through the filter and the filtrate is collected.

A.4.2.4 Addition of stabilizing agents and pH control agents (stabilizations)

The filtrate is adjusted with water and glycerol to the desired enzyme concentration. Raw materials are added as stabilising agents and pH control agents to enable the transportation of the filtrate between production sites, before final formulation. The exact raw materials used are listed in **Appendix 4.2**.

A.4.2.5 Sterile filtration

Before the bulk-product is leaving the production site for the final standardization and filling, the lipase passes a filter for the sterilization.

A.4.3 Standardisation and packaging

The food enzyme is formulated with ingredients and additives which are of food-grade quality. The enzyme preparation then undergoes a final sterile filtration and is packed.

A.5 Specification for identity and purity

The lipase enzyme product also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications (JECFA, 2006).

Analytical data for three representative, standardised batches of the lipase enzyme preparation are shown in (**Table 1**). These data show compliance with the purity criteria of the specification.

Table 1. Analytical data for three representative enzyme product batches

Control parameter	Unit	Specification	Batch 1	Batch 2	Batch 3
lipase activity	LVU/g		825	1174	1210
Lead	mg/kg	< 5	ND (LOQ < 0.05)	ND (LOQ < 0.05)	ND (LOQ < 0.05)
Escherichia coli	CFU/25 g	ND	ND	ND	ND
Total coliforms	CFU/g	< 30	<1	<1	<1
Salmonella spp.	CFU/25 g	ND	ND	ND	ND
Antimicrobial activity	—	ND	ND	ND	ND
Production strain	CFU/g	ND	ND	ND	ND

ND: not detected; LOQ: limit of quantification (*: LOQ is matrix-dependent); CFU: colony forming unit

The methods of analysis used to determine compliance with the specifications are enclosed (**Appendix 3**).

The mean total organic solids (TOS) of the three food enzyme batches is 1.3% and the mean enzyme activity/TOS ratio is 84.1 LVU/mg TOS.

The lipase enzyme preparation is available as a liquid enzyme product. The concentrate is standardised in lipase units (LVU/g; **Appendix 3**). The preparation does not contain known food allergens (**Appendix 2.1** and **Appendix 4.2**).

A.6 Analytical method for detection

The lipase enzyme preparation is to be used in the food industry as a processing aid. This information is not required in the case of an enzymatic processing aid.

B Information related to the safety of a chemical processing aid

Not applicable – this application does not concern a chemical processing aid.

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 enzyme is used as processing aid during processing of lipid-containing food in a range of countries, where there are no restrictions on the use of enzyme processing aids or where the enzyme is covered by a country positive list or specific approval.

The safety of the lipase preparation has been evaluated and confirmed by external expert groups, as follows:

- Denmark: The enzyme preparation was safety assessed resulting in the authorisation of the enzyme product by the Danish Veterinary and Food Administration.

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

(a) Information on the enzyme's prior history of human consumption and/or its similarity to proteins with a history of safe human consumption

A wide variety of enzymes are used in food processing. Enzymes, including lipase, have a long history of use in food (Pariza and Foster, 1983; Pariza and Johnson, 2001).

Lipase enzyme preparations from various sources are widely authorised in, e.g. Australia and New Zealand, Brazil, Canada, China, Denmark, France, Japan, and Mexico.

(b) Information on any significant similarity between the amino acid sequence of the enzyme and that of known protein toxins

A sequence homology assessment of the lipase enzyme to known amino acid sequences was conducted. The amino acid sequence of the lipase provided was used as input for the search. No homologies to known toxins were found. The complete search report is enclosed in **Appendix 5**.

As no issue of concern arise from the production strain (see section D1 and D2), it is considered that no toxicological testing other than assessment of allergenicity is necessary.

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

(a) Information of the source of the enzyme processing aid

The lipase enzyme is produced by a *K. phaffii* expressing the lipase from *Y. lipolytica*. *K. phaffii* is in general considered as a non-pathogenic organism (see Section 3.3.2 D).

(b) Analysis of similarity between the amino acid sequence of the enzyme and that of known allergens

The potential allergenicity of the enzyme produced with the genetically modified organism strain was assessed by comparing its amino acid sequence with those of known allergens

(**Appendix 5**). Using higher than 35% identity in a sliding window of 80 amino acids as the criterion, no matches were found.

No information is available on oral and respiratory sensitisation or elicitation reactions of this enzyme.

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

Documentation of approval of the lipase in Denmark is enclosed in **Appendix 2**.

D Additional information related to the safety of an enzyme processing aid derived from a microorganism

D.1 Information on the source microorganism

The lipase enzyme is produced by a *K. phaffi* expressing the lipase from *Y. lipolytica*. These yeasts of the *K.* genus are naturally found in tree exudates, with the first *P. pastoris* strains isolated from chestnut and oak trees (Heistinger et al., 2020; Phaff & Knapp, 1956).

The production strain for the lipase enzyme was developed from the *K. phaffii* CBS 7435 (synonymously NRRL Y-11430) which was then deposited under DSM 34125. This isolate has been the focus of much research, and many of the commonly used heterologous expression systems are derived from this progenitor strain (J. L. Cereghino & Cregg, 2000). This includes lipase produced by *K. phaffii* DSM 34125, as well as all the food enzymes/ingredients produced with *K. phaffii* (*P. pastoris*) that have been concluded safe for their intended use in Australia/New Zealand, the United States, or the European Union.

The lipase production strain is a non-pathogenic, non-toxigenic, genetically modified *K. phaffii* strain. The production strain does not produce secondary metabolites of toxicological concern to humans as explained in Section E 1.3, Section A.5 and **Appendix 6.1**.

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

K. phaffii (*P. pastoris*) has a history of safe use as a production organism for the expression of heterologous proteins (J. L. Cereghino & Cregg, 2000). Reports of infections implicated by *K. phaffii* (or closely related *Pichia* species) in humans are quite rare and opportunistic in nature (EFSA Scientific Committee, 2007; Liu et al., 2023). Accordingly, *K. phaffii (P. pastoris*) strains, including the CBS 7435 (NRRL Y-11430) host used to construct the production strain, are classified as a Biosafety Level-1 (BSL-1) organism by the ATCC, a category reserved for viable biological agents that are not known to consistently cause disease in healthy adult humans and requiring only minimal safety precautions in handling and storage (CDC, 2020).

Consistent with this viewpoint, *P. pastoris* has been included in EFSA's list of microorganisms with Qualified Presumption of Safety (QPS) status since this list was first established in 2007 (EFSA Scientific Committee, 2007). In the 2010 update of the QPS list, *P. pastoris* was reassigned as *K. pastoris* (EFSA BIOHAZ Panel, 2010), and *K. phaffii* was also later

recognized as a new taxonomic unit (EFSA BIOHAZ Panel, 2018ab, 2020). The QPS status of *K. phaffii* (and *K. pastoris*) continues to be maintained in the current list, with the recommendation only to use it for enzyme production, which implies the absence of viable cells of the production organism in the final product and can also be applied for food and feed products based on microbial biomass (EFSA BIOHAZ Panel, 2023a, 2023b). Although list of organisms with QPS status is granted at the species level for bacteria and yeast (EFSA BIOHAZ Panel, 2020; Herman et al., 2019), EFSA recognizes the QPS approach can be extended to genetically modified microorganisms, for which the species of the parental/recipient strain qualifies for the QPS approach and for which the genetic modifications do not give rise to safety concerns (EFSA BIOHAZ Panel, 2018ab, 2023b; EFSA CEP Panel, 2021b). Considering that *K. phaffii* (as a species) has QPS status, that the genetic modifications introduced are safe and well-characterized, and that no viable cells or DNA of the production strain remain in the lipase preparation, no safety concerns are anticipated from the use of the production strain as long as the strain is unambiguously species identified using up-to-date molecular methods.

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

The inserted recombinant DNA is genetically stable during fermentation, as the inserted DNA is integrated into the genome, and the strain does not harbour any plasmids or vectors that can be mobilized for genetic transfer to other organisms.

The phenotypic and genetic stability of the production strain is also demonstrated by its capacity to produce a consistent level of lipase enzyme. The enzyme activity level across representative batches (average 1070 LVU/g) have been presented in **Table 1**. Additionally, consistency in the production process is supported by the protein banding pattern observed from sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis (**Appendix 6.5**).

For a more detailed description of the strain construction and characteristics, please see section 3.3.2 E.

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

This section contains summarised information on the modifications of the host strain, on the content and nature of the introduced DNA and on the construction of the final production strain, as well as the stability of the inserted gene. The detailed information is provided in the confidential **Appendix 6**.

E.1.1 Host organism

The production strain for the lipase enzyme was developed from the *K. phaffii* CBS 7435 (synonymously NRRL Y-11430) which was then deposited under DSM 34125. This isolate has been the focus of much research, and many of the commonly used heterologous expression systems are derived from this progenitor strain (J. L. Cereghino & Cregg, 2000). This includes lipase produced by *K. phaffii* DSM 34125, as well as all the food enzymes/ingredients produced with *K. phaffii* (*P. pastoris*) that have been concluded safe for their intended use in Australia/New Zealand, the United States, or the European Union. The taxonomic classification of the strain is:

Phylum	Ascomycota
Class	Saccharomycetes
Order	Saccharomycetales
Family	Phaffomycetaceae
Genus	*Komagataella*
Species	*Komagataella phaffii*

E.1.2 Introduced DNA

Plasmids were used to transform the *K. phaffii* recipient strain.

Only the targeted gene expression cassettes from the plasmids are integrated to the production strain, and the other elements from the plasmids are not left in the production strain. One of the plasmids contains a lipase expression cassette consisting of a promoter, the coding sequence for lipase from *Y. lipolytica* and a terminator.

E.1.3 Construction of the recombinant microorganism

The production strain was then constructed by genetic modifications. Further details can be found in **Appendix 6.1**.

E.1.4 Antibiotic resistance gene

No functional antibiotic resistance genes were left in the strain as a result of the genetic modifications. Further details can be found in **Appendix 6.1**.

E.1.5 Stability of the introduced genetic sequences

The inserted recombinant DNA is genetically stable during fermentation, as the inserted DNA is integrated into the genome, and the strain does not harbour any plasmids or vectors that can be mobilized for genetic transfer to other organisms.

The phenotypic and genetic stability of the production strain is also demonstrated by its capacity to produce a consistent level of lipase enzyme. The enzyme activity level across representative batches (average 1070 LVU/g) have been presented in **Table 1**. Additionally, consistency in the production process is supported by the protein banding pattern observed

from sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis (**Appendix 6.5**).

F Information related to the dietary exposure to the processing aid

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

The lipase preparation is used as a processing aid during the manufacture of lipid-based products. Triacylglycerol lipases catalyse the hydrolysis of triglycerides, diglycerides and monoglycerides to yield free fatty acids and monoglycerides, diglycerides and glycerol.

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

The lipase enzyme preparation is used in the following food manufacturing processes:

- cheese production,
- production of flavouring preparations from dairy products (enzyme-modified dairy ingredients),
- production of plant-based analogues of milk and milk products.

Use level

The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following GMP.

The conditions of use of the lipase preparation during food processing do not only depend on the type of application, but also on the food production process of each individual food manufacturer. In order to ensure optimal effectiveness of the enzyme at an acceptable economic cost the dosage, reaction time, process conditions and processing steps are adjusted.

The highest dosage given for cheese production is 100 LVU per litre of milk. This corresponds to 1.2 mg TOS per kg of raw material. Typically, 10 L of milk is required to produce 1 kg of cheese. As a conservative measure, a 10-fold conversion factor was applied, which assumes all of the enzyme is carried forward without partition between the curd and whey fractions. In reality, it is estimated that only ~20% of the lipase added during milk processing remains in the cheese, whereas ~80% of the enzyme will end up in the whey fraction (EFSA CEP Panel, 2023). Therefore, the maximum levels of the lipase preparation that could theoretically remain in foods and beverages processed with this enzyme is 12 mg TOS/kg final food.

Enzyme residues in the final food

The lipase preparation is used during processing and does not exert any enzymatic activity in the final foods that are ingested by consumers. This is due to a combination of various factors, and is dependent on the process conditions used by the individual food producer. These

factors can include: denaturation of the enzyme during heat processing (e.g., pasteurization, UHT treatment), depletion of the substrate, pH changes, digestion by proteases from the starter cultures and any additional ripening cultures, physical removal of the enzyme, etc. Therefore, the enzyme will not exert any significant technical effects in or on final food products.
Estimates of human consumption

Method used for the dietary exposure assessment

The Budget Method was used to estimate the potential dietary exposure resulting from the intended uses of lipase in food processing. The Budget Method is a preliminary screening tool developed to assess the intake of food additives (FAO/WHO, 2020), and it is widely accepted and used for the derivation of dietary exposure estimates for food enzymes. The Budget Method provides an estimate of the Theoretical Maximum Daily Intake (TMDI), and it relies upon conservative assumptions regarding: 1) the level of consumption of foods and beverages; 2) the proportion of foods and beverages that may contain the food enzyme; and 3) the maximum concentration of the food enzyme in foods and beverages.

1) Level of consumption of foods and beverages

In the FAO/WHO's Principles and methods for the risk assessment of chemicals in food - Chapter 6: Dietary Exposure Assessment of Chemicals in Food (FAO/WHO, 2020), the Budget Method considers the level of consumption for foods and beverages to be the maximum physiological level of consumption that is theoretically possible. Specifically, it is assumed that individuals would maximally consume 0.05 kg of solid food per kg of body weight (bw) per day, and 0.1 liter of beverage per kg bw per day. For a 60 kg individual, these levels correspond to the consumption of 3 kg of food and 6 liters of beverages per day.

2) Proportion of foods and beverages that may contain the food enzyme

According to the FAO/WHO report (FAO/WHO, 2020), it can be assumed that 12.5% of solid foods and 25% of beverages contain the food additive (i.e., food enzyme in this case). For additives (enzymes) that are used in a wide range of foods, the proportion of solid foods containing the additive may be set at 25%.

3) Maximum concentration of the food enzyme in foods and beverages

For the exposure assessment, it is assumed the lipase preparation added during processing will remain in the final foods as consumed, and that no removal and/or inactivation of the enzyme occurs. Therefore, the maximum levels of the lipase preparation that could theoretically remain in foods and beverages processed with this enzyme is 12 mg TOS/kg final food, as explained above.

TMDI (Theoretical Maximum Daily Intake)

The TMDI of the lipase preparation from its intended uses in the processing of foods was calculated as 0.45 mg TOS/kg bw/day, as presented in **Table 2**. Because of the conservative nature of the assumptions underlying the Budget Method, this TMDI is considered to be a gross overestimation of the potential dietary exposure that could occur in practice. Specifically, it is conservatively assumed that:

- A quarter (25%) of all foods and beverages that can be physiologically consumed per day (i.e., 3 kg of solid foods and 6 liters of beverages for a 60 kg individual) will require the use of lipase during its production, or contain ingredients that were processed with lipase.
- The aforementioned foods and beverages specifically contain lipase from *Y. lipolytica* expressed in *K. phaffii*. In reality, it is highly unlikely that this specific lipase will have 100% market share, and other lipase preparations produced by Chr. Hansen or other suppliers could be used.
- The lipase will be added at the highest anticipated use level during processing. In practice, the enzyme will be added only at levels that are required to achieve the desired technical effects, and some applications may require less enzyme than the maximum use level employed in the TMDI calculation.
- Foods with residual lipase contain the highest theoretical level of enzyme that could be carried over (i.e., 12 mg TOS/kg final foods), with no removal or inactivation of the enzyme during the manufacturing steps for these food products.

Table. 2 Theoretical Maximum Daily Intake (TMDI) from the Intended Uses of Lipase from *Y. lipolytica* Expressed in *K. phaffii*

Type of Product	**(A)**	**(B)**	**(C)**	**(D)**	**TMDI (mg TOS/kg bw/day)**
	Level of consumption (kg/kg bw/day)	**Proportion of foods or beverages with lipase (%)**	**Consumption level of foods or beverages with lipase (kg/kg bw/day)**	**Maximum use level of lipase in foods or beverages (mg TOS/kg)**	
Solid foods	0.05	25	0.0125	12	0.15
Beverages	0.1	25	0.025	12	0.30
Total					**0.45**
Calculations: (C) = (A) x (B/100). TMDI = (D) x (C).					

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 relevant.

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

It is assumed that all raw materials containing lipid are processed using the lipase object of this submission as a processing aid at the highest recommended dosage.

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

As described in F.2.1 above, a "worst case" calculation is made assuming that all organic matter originating from the enzyme is retained in the processed food product. The dietary exposure is estimated using the Budget Method, as the processed lipid are used as an ingredient in a variety of food products.

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

No significant changes in recent years are observed.

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List of appendices

Appendices listed in **red, bold** font should be treated as confidential commercial information.

Appendix 1.1 Formal request for treatment of confidential commercial information (CCI)

Appendix 1.2 Checklist for general requirements

Appendix 1.3 Checklist for applications for substances added to food

Appendix 2.1 Product data sheet

Appendix 2.2 Danish approval document

Appendix 3 Methods of Analysis

Appendix 4.1 FSSC 22000 certificate

Appendix 4.2 CCI Raw materials used during fermentation and recovery

Appendix 4.2 non-CCI Raw Materials used during fermentation and recovery

Appendix 5 Sequence homology assessment

Appendix 6 CCI Documentation regarding the production strain

Appendix 6 non-CCI Documentation regarding the production strain