AB Enzymes GmbH – Feldbergstrasse 78 , D-6412 Darmstadt

A Petition to Amend the Australia New Zealand Food Standards Code with a Triacylglycerol Lipase Enzyme Preparation produced by *Trichoderma reesei*

AB Enzymes GmbH

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II. EXECUTIVE SUMMARY

The present application seeks to schedule 18 - Processing Aids of the Australia New Zealand Food Standards Code (the Code) to approve a triacylglycerol lipase enzyme preparation from *Trichoderma reesei* produced by AB Enzymes GmbH for use as a processing aid in the manufacturing of cereal based products.

Proposed change to Standard 1.3.3 - Processing Aids

The table schedule 18—9(3), Permitted processing aids various purposes, is proposed to be amended to include a genetically modified strain of *Trichoderma reesei* as permitted source for triacylglycerol lipase (EC 3.1.1.3).

This application is submitted under a general assessment procedure.

Description of Enzyme Preparation

The food enzyme is a biological isolate of variable composition, containing the enzyme protein, as well as organic and inorganic material derived from the microorganism and fermentation process.
The main activity of the food enzyme is triacylglycerol lipase (EC 3.1.1.3).

Lipase hydrolyses ester bonds of triacylglycerols, resulting in the formation of mono- and diacylglycerols, free fatty acids and, in some cases, also glycerol. Lipases can be divided into four groups according to their specificity: substrate specific lipases, regioselective lipases, fatty acid specific lipases and stereospecific lipases.

The substrates for lipase are non-polar lipids such as triglycerides or triacylglycerol.

Triglycerides are formed by combining glycerol with three fatty acids molecules. The glycerol molecule has three hydroxyl (OH-) groups. Each fatty acid has a carboxyl group (COOH-). In triglycerides, the hydroxyl groups of the glycerol join the carboxyl groups of the fatty acid to form esters bonds:

HOCH2CH(OH)CH2OH + RCO2H + R'CO2H + R''CO2H → RCO2CH2CH(O2CR')CH2CO2R'' + 3H2O

Triglycerides are founds in plants and animals: they are the main constituents of vegetable oils and animal fats (Nelson et al., 2000). They are a major component of human skin oil (Lampe et al., 1983). Triglycerides and triacylglycerols are also found in wheat flour: wheat flour contains approximately 2.0–2.5% lipids; wheat lipids can be divided into polar (glycolipids, phospholipids) and non-polar lipids (triacylglycerides, mono-glycerides), as shown in the Figure 1 below:

Tri
Mono

It should be noted that the production organism is removed during filtration and is not present in the final enzyme preparation.

Use of the Enzyme

In general, the technological need of the enzymatic conversion of triglycerides with the help of triacylglycerol lipase can mainly be described as the degradation of a component (the substrate triglycerides).

Triacylglycerol lipase can be used in Cereal-based products (as of the Australian Food Code) such as, but not limited to, bread, biscuits, steamed bread, cakes, pancakes, tortillas, wafers and waffles. Non polar lipids such as triglycerides and triacylglycerols are found in wheat flour: wheat flour contains

approximately 2.0–2.5% lipids; wheat lipids can be divided into polar (glycolipids, phospholipids) and non-polar lipids (triacylglycerides, mono-glycerides).

Food enzyme preparations are used by food manufacturers according to the Quantum Satis principle, which means that food manufacturers will typically fine-tune the enzyme dosage based on a dose range recommended by the enzyme supplier.

Benefits

Triacylglycerol lipase from *Fusarium oxysporum* expressed in *T.reesei* is mainly intended to be used in baking processes, (e.g. bread, biscuits, tortillas, cakes, steamed bread and croissants) and other cereal based processes (e.g. pastas, noodles and snacks).

The use of lipase can therefore influence the interactions between the different constituents of the dough, i.e. gluten proteins and lipids, starch and lipids as well as gluten and starch. The benefits of the conversion of triglycerides (non-polar lipids) with the help of lipase in baking can therefore be summarized as follows:

- Facilitate the handling of the dough
- Improve dough stability and strength which results in processing tolerance
- Improve the dough's structure and behaviour during the baking steps
- Regulate batter viscosity, beneficial in the production process for e.g. waffles, pancakes and biscuits

The benefits of the conversion of the triglycerides (non-polar lipids) with the help of lipase in other cereal based processes can be summarized as follows:

- Facilitate the handling of the dough
- Improve dough stability and strength which results in processing tolerance
- Reduce oil uptake during frying

Safety Evaluation

The food enzyme object of the present dossier was subjected to several toxicological studies to confirm its safety for consumers. The mutagenicity studies showed that the food enzyme does not have the potential to damage the genetic material of living organisms, including mammals. The oral toxicity study showed that the food enzyme does not exhibit signs of toxicity, up to doses that are several thousand times higher than those which are consumed via food.

The product complies with the recommended purity specifications (microbiological and chemical requirements) of the FAO/WHO's Joint Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC) for food-grade enzymes.

The product is free of production strain and recombinant DNA.

The safety of the Triacylglycerol lipase preparation was confirmed or is under consideration by external expert groups, as follows:

- **France**: The enzyme preparation was safety assessed according to the Guidelines for the evaluation of food enzymes. This resulted in the authorisation of the enzyme product by the French authorities in April 2017.
- **USA**: A GRAS no objection letter determined that the xylanase enzyme reparation is GRAS for its intended use GRAS #631
- **EFSA/ EU Commission**: A dossier was submitted in 2015 in compliance with Regulation (EC) 1332/2008 and is currently being reviewed by EFSA.

Conclusion

Based on the safety evaluation, AB Enzymes GmbH respectfully request the inclusion of triacylglycerol lipase from *Fusarium oxysporum* expressed in *T. reesei* in the table – 18-9(3) of schedule 18 - Permitted processing aids various purposes.

III. INTRODUCTION

The dossier herein describes a *Trichoderma reesei* produced triacylglycerol expressing a gene from *Fusarium oxysporum* produced by submerged fermentation.

Triacylglycerol lipase from *Fusarium oxysporum* expressed in *T. reesei* is mainly intended to be used in baking processes, (e.g. bread, biscuits, tortillas, cakes, steamed bread and croissants) and other cereal based processes (e.g. pastas, noodles and snacks).

The following sections describe the genetic modifications implemented in the development of the production microorganism to create a safe standard host strain resulting in a genetically well-characterized production strain, free from harmful sequences.

Further sections show the enzymatic activity of the enzyme, along with comparison to other similar enzymes. The safety of the materials used in manufacturing, and the manufacturing process itself is described. The hygienic measurements, composition and specifications as well as the self-limiting levels of use for triacylglycerol lipase are described. Information on the mode of action, applications, and use levels and enzyme residues in final food products are described. The safety studies outlined herein indicate that the triacylglycerol enzyme preparation from *T. reesei* shows no evidence of pathogenic or toxic effects. Estimates of human consumption and an evaluation of dietary exposure are also included.

IV. Section 3.1, GENERAL REQUIREMENTS

3.1.1. Executive Summary

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

3.1.2. Applicant Details

Applicant's name
AB Enzymes GmbH

Company
AB Enzymes GmbH
Feldbergstr. 78
D-64293 Darmstadt
Germany

Telephone Number

Email Address

Nature of Applicant's Business
Biotechnology

Dossier prepared by

AB Enzymes GmbH
Feldbergstr. 78
D-64293 Darmstadt
Germany

3.1.3. Purpose of the Application

The table (section 1.3.3—11) 18—9(3), Permitted processing aids various purposes, is proposed to be amended to include a genetically modified strain of *Trichoderma reesei* as permitted source for triacylglycerol lipase (EC 3.1.1.3).

3.1.4. Justification for the Application

The need for the proposed change:

Trichoderma reesei expressing a triacylglycerol lipase gene from *Fusarium oxysporum* is not present as an approved source in the table to schedule 18 of standard 1.3.3.; Permitted Enzymes of Microbial Enzymes. AB Enzymes GmbH is requesting that this source organism be added. See 3.1.5 for details regarding the advantages of the proposed change.

3.1.5. The Advantages of the Proposed Change over the Status Quo:

The lipase enzyme is one of AB Enzymes latest achievements and has showed great potential in food manufacturing as detailed in this customer support letter, Appendix #1.1.

The enzymes known in the art and listed in standard 3.1.1 as current status quo derived from other sources have technical limitations, especially with regards to processing (tolerance to withstand mechanical shock during process). Based on market benchmarking we have found that our product has superior technical characteristics resulting in improved quality for bakery product manufacturers. This is a characteristic that is strongly preferred by manufacturers. There is also a cost benefit associated with the use of *Trichoderma reseei* as superior producer of enzymes resulting in a cost benefit that is passed on to the final user of the enzyme. Increased competition on the market is also a desired characteristic in the context of competition laws. It will increase the choice to local manufacturers and help in reducing production costs as compared to the currently known and marketed products of the same enzyme class used for the same type of bread making applications.

Due to the effectiveness of this enzyme in the above-mentioned food processes, AB Enzymes has received authorization to sell in both USA and France. Applications have been submitted globally and are currently under review.

Furthermore there are no public health or safety issues related to the proposed change.

3.1.6. Regulatory Impact Statement:

The addition of the enzyme to Schedule 18-9(3) is not intended to place any costs or regulatory restrictions on industry or consumers. Inclusion of the enzyme will provide food manufacturers with an alternative. For government, the burden is limited to necessary activities for a variation of Standard 1.3.3.

3.1.7. Impact on International Trade:

There will be a positive impact on Australia / New Zealand manufacturers of bakery products and bakery mixes. Many of these companies are active in export markets of Southeast Asia or the Middle East and are facing local competition and competitors from Europe or North America. Many of the competitors have already access to these new tools and their beneficial cost/performance. The approval of the enzyme could therefore have a positive impact to keep Australia / New Zealand manufacturers competitive in international trade.

3.1.8. Information to Support the Application

Public Health and Safety Issues related to the Proposed Change:

No public health and safety issues are expected from the proposed changes.

The food enzyme object of the present dossier was subjected to several toxicological studies to confirm its safety for consumers. The genotoxicity studies showed that the food enzyme does not have the potential to damage the genetic material of living organisms, including mammals. The oral toxicity study showed that the food enzyme does not exhibit signs of toxicity, up to doses that are several thousand times higher than those which are consumed via food.

The product complies with the recommended purity specifications (microbiological and chemical requirements) of the FAO/WHO's Joint Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC) for food-grade enzymes.

The product is free of production strain and recombinant DNA.

Consumer choice related to the Proposed Change:

Consumer choice is not expected to be changed directly as the enzyme is used as a processing aid and is not purchased by consumers. Triacylglycerol lipase does not perform any technological function in the final foods containing ingredients prepared with the help of this enzyme. Moreover, the food products prepared with the help of triacylglycerol lipase do not have other characteristics than what is expected by the consumer. Consumers could be impacted indirectly by companies able to pass cost savings from utilizing enzymes in food processing on to their customers.

3.1.9. 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 characterized as "General Procedure, Level 1".

3.1.10. Confidential Commercial Information (CCI)

Detailed information on the construction and characteristics of the genetically modified production strain is provided in the confidential Appendix #13. A summary of this information is given in section E of section 3.2.2. The formal request for treatment of Appendix #13 as confidential commercial information (CCI) is included as Appendix #1.2.

3.1.11. Other Confidential Information

Information related to the methods used to analyze enzymatic activity is company specific and this information is not publically available and known only to AB Enzymes GmbH, as such we respectfully ask that this information is kept confidential as presented in Appendix #2. The formal request for treatment of Appendix #2 as other confidential information is included as Appendix #1.3.

3.1.12. Exclusive Capturable Commercial Benefit (ECCB)

This application is not expected to confer an Exclusive Capturable Commercial Benefit, as once the enzyme and source organism is listed publically on FSANZ website, any company can benefit from the use of the enzyme.

3.1.13. International and other National Standards

International Standards:

Use of enzymes as processing aids in cereal and cereal-based products and is not restricted by any Codex Alimentarius Commission (Codex) Standards or any other known regulations.

National Standards:

Use of enzymes as processing aids in food applications (baking, brewing, starch processing, etc.) has specific standard in France (arrêté du 19 octobre 2006[1]), and the use of this enzyme has been approved for the accordant food applications in this dossier (please see **Section C.1**).

In the USA, under CFR, Code of Federal Regulations, title 21, [21CFR170.3], Food for Human Consumption, Food additives - *Enzymes* : Enzymes used to improve food processing and the quality of the finished food.

Lipase is listed on the Food Additive Index of CODEX General Standard for Food Additives (GSFA) (INS: 1104). Also, this food enzyme, triacylglycerol lipase, complies with the internationally accepted JECFA specifications for chemical and microbiological purity of food enzymes (FAO/WHO, 2006).

3.1.14. Statutory Declaration

The Statutory Declaration is included as Appendix #1.4.

This application concerns an enzyme product intended to be used as a processing aid for food manufacturing.

Therefore, the relevant documentation according to the Application Handbook from Food Standards Australia New Zealand as of March 1, 2016, are the following sections:

- SECTION 3.1 – GENERAL REQUIREMENTS
- SECTION 3.3.2 – PROCESSING AIDS, subsections A, C, D, E, F

[1] https://www.legifrance.gouv.fr/affichTexte.do?cidTexte=LEGITEXT000020667468

Accordingly, the checklist for General Requirements as well as the Processing Aids part of the checklist for Standards related to Substances added to Food was used and is included as Appendix #1.5.

V. Section 3.3.2. STANDARDS RELATED TO SUBSTANCES ADDED TO FOOD PROCESSING AID

A. Technical Information of the Processing aid

A.1. Information on the type of processing aid

This dossier includes a triacylglycerol lipase enzyme, produced with the help of *Trichoderma reesei* strain RF10625. The representative current commercial product is Veron® Hyperbake ST.

Triacylglycerol lipase is a microbial produced enzyme and already belongs to the table to Schedule 18 of standard 1.3.3.; Permitted enzymes of Microbial Enzymes.

Enzyme preparations are generally used *quantum satis*. The average dosage of the enzyme depends on the application, the type and quality of the raw materials used, and the process conditions. This dossier is specifically submitted for use of triacylglycerol lipase used in baking processes and other cereal-based processes. A further description of the enzyme in these food technology applications will be given in subsequent sections.

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

A.2.1.Enzyme

Systematic name	Triacylglycerol acylhydrolase [2]
Common names	Triacylglycerol lipase, lipase; triglyceride lipase; tributyrase; butyrinase; glycerol ester hydrolase; tributyrinase; Tween hydrolase; steapsin; triacetinase; tributyrin esterase; Tweenase; amno N-AP; Takedo 1969-4-9; Meito MY 30; Tweenesterase; GA 56; capalase L; triglyceride hydrolase; triolein hydrolase; tween-hydrolyzing esterase; amano CE; cacordase; triglyceridase; triacylglycerol ester hydrolase; amano P; amano AP; PPL; glycerol-ester hydrolase; GEH; meito Sangyo OF lipase; hepatic lipase; lipazin; post-heparin plasma protamine-resistant lipase; salt-resistant post-heparin

[2] Lipase and triacylgycerol lipase will be used interchangeable in this dossier.

	lipase; heparin releasable hepatic lipase; amano CES; amano B; tributyrase; triglyceride lipase; liver lipase; hepatic monoacylglycerol acyltransferase
Enzyme Commission No.	EC 3.1.1.3
CAS number	9001-62-1
Host	*Trichoderma reesei*
Donor	*Fusarium oxysporum*

A.2.2.Enzyme Preparation

The commercial names representative of the enzyme preparation, formulated with the enzyme produced with RF10625 *T. reesei*, is Veron® Hyperbake ST. The product data sheets are provided in Appendix #1.

A.2.3.Enzyme preparation composition:

Composition Veron® Hyperbake ST	
Lipase	8.8%
Sunflower oil	0.4%
Wheat flour	90.8%

The main activity of the enzyme preparation is lipase (IUB 3.1.1.3), which has been identified in many sources, including plants, microorganisms and animals.

Lipase hydrolyses ester bonds of triacylglycerols, resulting in the formation of mono- and diacylglycerols, free fatty acids and, in some cases, also glycerol. Lipases can be divided into four groups according to their specificity: substrate specific lipases, regioselective lipases, fatty acid specific lipases and stereospecific lipases.

The substrates for lipase are non-polar lipids such as triglycerides or triacylglycerol.

Triglycerides are formed by combining glycerol with three fatty acids molecules. The glycerol molecule has three hydroxyl (OH-) groups. Each fatty acid has a carboxyl group (COOH-). In triglycerides, the hydroxyl groups of the glycerol join the carboxyl groups of the fatty acid to form esters bonds:

$$HOCH_2CH(OH)CH_2OH + RCO_2H + R'CO_2H + R''CO_2H \rightarrow RCO_2CH_2CH(O_2CR')CH_2CO_2R'' + 3H_2O$$

Triglycerides are founds in plants and animals: they are the main constituents of vegetable oils and animal fats. They are a major component of human skin oil (Lampe et al. 1983). Triglycerides and triacylglycerols are also found in wheat flour: wheat flour contains approximately 2.0–2.5% lipids; wheat lipids can be divided into polar (glycolipids, phospholipids) and non-polar lipids (triacylglycerides, mono-glycerides), as shown in the Figure 1 below:

Figure 1. Classification of wheat flour lipids

Consequently, the substrate for lipase occurs naturally in nature and is therefore a natural part of the human diet.

Reaction products: as a result of the catalytic activity of lipase low levels of mono- and diacylglycerols, free fatty acids and, in some cases also glycerol, are formed. These compounds are also natural constituents in various organisms from bacteria to mammals and are consequently already present in the human diet.

The method to analyse the activity of the enzyme is company specific and is capable of quantifying lipase activity as defined by its IUBMB classification. The enzyme activity is usually reported in *ALU*/g^{-1} (Appendix #2, listed as "other" confidential information).

A.2.4.Enzyme genetic modification

The enzyme is from a *Trichoderma reesei* host strain genetically modified with a lipase gene deriving from *Fusarium oxysporum*. The enzyme is not considered protein engineered.

Name of the enzyme protein:	**Triacylglycerol lipase**
Donor:	*Fusarium oxysporum*
Host:	*Trichoderma reesei*
Production strain:	*Trichoderma reesei* RF10625

For more detailed information on the genetic modification, please see **Section E**.

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

Product –Veron® Hyperbake ST

Properties	
pH Value	7-9
Density	1.00-1.10 g/ml
Appearance	Light beige colour with characteristic odour.

The substrates and the reaction products are themselves present in food ingredients. 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. Consequently, no adverse effect on nutrients is expected.

Like most of the enzymes, lipase performs its technological function during food processing and does not perform any technological function in the final food. The reasons why the enzyme does not exert any (unintentional) enzymatic activity in the final food can be due to a combination of various factors, depending on the application and the process conditions used by the individual food producer. These factors include depletion of the substrate, denaturation of the enzyme during processing (which is clearly the case during baking process), lack of water activity, wrong pH, etc. In some cases (e.g. after alcohol distillation, products resulting from starch processing), the enzyme may no longer be present in the final food.

Based on the conditions of use described in **Section F** and the activity of lipase under such conditions, it can be concluded that the enzyme lipase does not exert any (unintentional) enzymatic activity in final bakery products.

Please refer to product data sheets for shelf-life and storage conditions.

For the Chemical properties – see **Section A.5**.

A.4. Information on the technological need and mechanism of action of the enzyme in food

In general, the technological need of the enzymatic conversion of triglycerides with the help of lipase can mainly be described as the degradation of a component (the substrate triglycerides).

As described above, lipase is naturally present in many animal and vegetable raw materials, including wheat grains. The natural enzymatic conversion of triglycerides in such materials would theoretically be of technological benefit in several industrial food manufacturing processes. However, the levels of endogenous lipase are often inadequate and vary from batch to batch of raw material, and the specificity of the enzyme may not be optimal to give desired process advantages. Therefore, industrial

lipase is used during food processing. Typical uses of lipase in food processing are dairy processing, fats and oils processing, meat and fish processing, beverage industry and baking. In those processes, the lipase is used as a processing aid in food manufacturing and is not added directly to final foodstuffs.
This dossier is specifically submitted for the use of lipase in baking (e.g. bread, biscuits, tortillas, cakes, steamed bread and croissants) and other cereal based processes (e.g. pastas, noodles and snacks). Lipases have been used in baking for the last 30 year and their use in the bakery industry is continuously increasing. This application has been specifically approved for a number of years in Denmark and France (including the "Pain de tradition Française"), which –together with the extensive use for decades in a number of EU countries- demonstrates the technological need of lipases in these food processes.

Below, the benefits of the use of industrial lipase in those processes are described. The beneficial effects are of value to the food chain because they lead to better and/or more consistent product characteristics. Moreover, the applications lead to more effective production processes, resulting in better production economy and environmental benefits such as the use of less raw materials and the production of less waste.

During mixing, wheat flour free lipids become bound or trapped within the gluten fraction. Limited hydrolysis of the triglycerides with the help of lipase results in an improved natural ratio of polar lipids. Increased proportion of polar lipids has a positive effect on gas retention, as they can align at the interface of the gas cells formed in the dough and therefore increase the stability of the gas cells, whereas endogenous wheat non-polar lipids destabilise gas cells in dough and therefore limit bread volume. The use of lipase helps removing this negative effect.

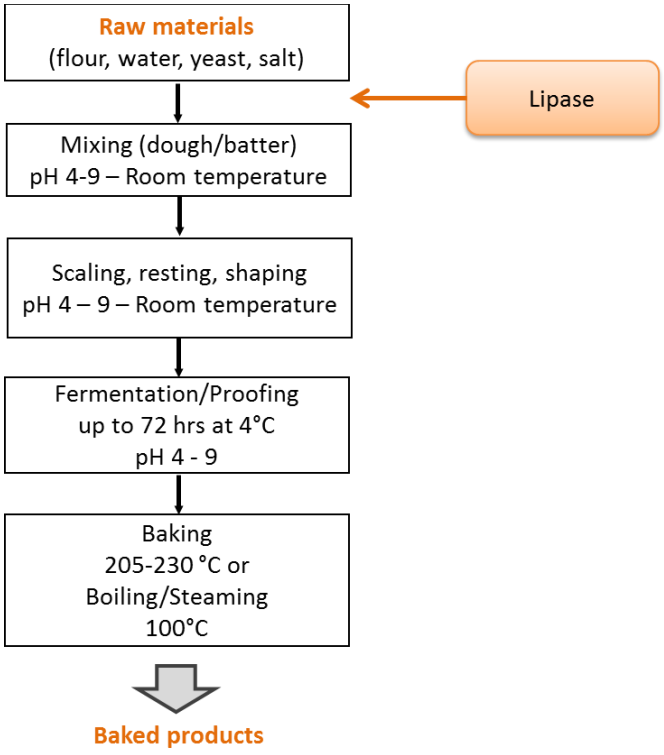
In addition, the degradation of the substrate triglycerides with the help of lipase leads to the creation of monoacyl-glycerol, that interacts with gelatinizing starch, in particular with amylose to form irreversible monoacyl-glycerol-amylose complexes.

The use of lipase can therefore influence the interactions between the different constituents of the dough, i.e. gluten proteins and lipids, starch and lipids as well as gluten and starch. The benefits of the conversion of triglycerides (non-polar lipids) with the help of lipase in baking can therefore be summarized as follows:

- Facilitate the handling of the dough
- Improve dough stability and strengh which results in processing tolerance
- Improve the dough's structure and behaviour during the baking steps
- Regulate batter viscosity, beneficial in the production process for e.g. waffles, pancakes and biscuits

Lipase is acting on one family of the components of the plant cell wall, and is often used together with other enzymes (enzyme systems) which modify other components of the plant cell walls. In particular, lipase is often applied together with endo-amylase, xylanase and cellulase (e.g. for brewing, grain processing, starch processing and beverage alcohol processing).

The process flow of baking process is presented below:



The fate of the enzyme protein during baking :

In baking, lipase performs its technological function during dough or batter handling in order to contribute to an improved and consistent baking process. The lipase is denatured by heat during the baking or steaming step.

Other cereal based processes:

Lipase can also be used in the processing of other cereal based products such as, but not limited to, pasta, noodles and snacks.

Lipids provide functional properties during pasta, noodle and snack making - due to their ability to interact with gluten and the water phase. Limited hydrolysis of lipids with the help of lipase improves the functional properties of the flour endogenous lipids, as explained below.

Dried pasta has, among cereal derived foodstuffs, a very distinct microscopic structure. It has a continuous protein mixture phase (the gluten or the protein network) wherein the starch granules are dispersed. While cooking in hot water, the starch granules gelatinize, i.e. absorb water, swell and turn into starch paste. The gluten (the protein network) is denatured through cooking and if it is not sufficiently resistant, the starch granules, when swelling, can tear the meshes of its continuous phase, thereby giving rise, at the periphery of the pasta, to a viscous layer of starch paste.

The state of the protein network after cooking can also affect the elasticity of the pasta. The main problem which has to be solved to obtain elastic and non-sticky pasta thus consists in increasing the resistance of the protein network to cooking (USA, 1970).

Pasta treated with lipase show higher amylose-lipid melting enthalpies (increase of around 75% more melting enthalpy in the cooked pasta treated by lipase), indicating that hydrolysis products of lipase do form complexes with amylose during cooking. These complexes inhibit the swelling of starch and the leakage of amylose during cooking, resulting in a firmer texture and smoother surface. Further, the

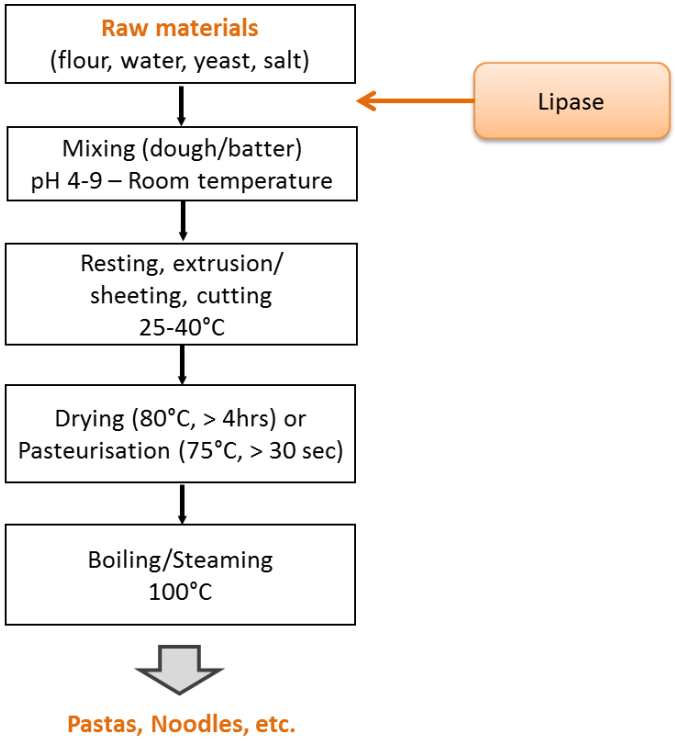
complex-building capability of the lipase hydrolysis products with amylose reduces leaching of amylose, resulting in less stickiness of products (VTT Biotechnology and TNO symposium, 2007).

Because gluten has a predominant role in the structure, the use of lipase, by increasing the gluten protein network resistance to cooking also plays a role in reducing the porosity and oil uptake during (noodles) frying (Gulia *et al*., 2014).

Therefore, the benefits of the conversion of the triglycerides (non polar lipids) with the help of lipase in other cereal based processes can be summarized as follows:

- Facilitate the handling of the dough
- Improve dough stability and strengh which results in processing tolerance
- Reduce oil uptake during frying

The process flow of other cereal based processes is presented below:



The fate of the enzyme protein during the pasta and noodles production process:

In other cereal based processes such as pasta and noodles, the lipase performs its function during dough handling. The enzyme is denatured by heat during the drying, boiling or steaming step.

The use of lipase in such applications has been specifically approved in France, which - together with the extensive use for decades in a number of EU countries and in the rest of the world - demonstrates the technological need of such food enzymes in food processes.

A.5. Manufacturing Process

Like all food enzymes, lipase described in this dossier is manufactured in accordance with current Good Manufacturing Practices for Food (cGMPs) and the principals of Hazard Analysis of Critical Control Points (HACCP). Compliance to Food Hygiene Regulation is regularly controlled by relevant food inspection services in Finland. Quality certificates are provided in Appendix #3.

T. reesei RF10625 described herein is produced by controlled submerged fermentation. The production process involves the fermentation process, recovery (downstream processing) and formulation and packaging. A manufacturing flow-chart is given in Appendix #4.

It should be noted that the fermentation process of microbial food enzymes is substantially equivalent across the world. This is also true for the recovery process: in a vast majority of cases, the enzyme protein in question is only partially separated from the other organic material present in the food enzyme.

A.5.1.Fermentation

The lipase enzyme is produced by submerged fermentation of the genetically modified strain of *Trichoderma reesei*. Please see **Section E** for a more detailed description of the genetic modification.

The production of food enzymes from microbial sources follows the process involving fermentation as described below. Fermentation is a well-known process that occurs in food and has been used for the production of food enzymes for decades. The main fermentation steps are:

- Inoculum
- Seed fermentation
- Main fermentation

A.5.2.Raw materials

The raw materials used in the fermentation and recovery processes are standard ingredients that meet predefined quality standards controlled by Quality Assurance for ROAL Oy. The safety is further confirmed by toxicology studies (See **Section C**). The raw materials conform to either specifications set out in the Food Chemical Codex, 10th edition, 2016 or The Council Regulation 93/315/EEC, setting the basic principles of EU legislation on contaminants and food, and Commission Regulation (EC) No 1881/2006 setting maximum limits for certain contaminants in food.

The raw materials used for the formulation are of food grade quality.

A.5.3. Materials used in the fermentation process (inoculum, seed and main fermentation)

- Potable water
- A carbon source (e.g. glucose, …)
- A nitrogen source (e.g. wheat derived material, …)
- Salts and minerals (e.g. Ammonium sulphate, Monopotassium phosphate)
- pH adjustment agents
- Foam control agents (e.g. polyalkylene glycols)

For details regarding fermentation ingredients please refer to Appendix #13-CCI.

A.5.4. Inoculum

A suspension of a pure culture of *T. reesei* RF10625 is aseptically transferred to a shake flask (1 liter) containing fermentation medium.

In order to have sufficient amount of biomass, the process is repeated several times. When a sufficient amount of biomass is obtained the shake flasks are combined to be used to inoculate the seed fermentor.

A.5.5. Seed fermentation

The inoculum is aseptically transferred to a pilot fermentor and then to the seed fermentor. The seed fermentation is run at a constant temperature and a fixed pH. At the end of fermentation, the inoculum is aseptically transferred to the main fermentation.

A.5.6. Main fermentation

Biosynthesis of the lipase enzyme product by the production strain *T. reesei* RF10625 occurs during the main fermentation.

The content of the seed fermentor is aseptically transferred to the main fermentor containing fermentation medium. The fermentation in the main fermentor is run as normal submerged fermentation under well-defined process conditions (pH, temperature, mixing, etc.).

The fermentation process is continued for a predetermined time or until laboratory test data show that the desired enzyme production has been obtained or that the rate of enzyme production has decreased below a predetermined production rate. When these conditions are met, the fermentation is completed.

A.5.7.Recovery

The purpose of the recovery process is:

- to separate the fermentation broth into biomass and fermentation medium containing the desired enzyme protein,
- to concentrate the desired enzyme protein and to improve the ratio enzyme activity/Total Organic Substance (TOS).

During fermentation, the enzyme protein is secreted by the producing microorganism into the fermentation medium. During recovery, the enzyme-containing fermentation medium is separated from the biomass.

This Section first describes the materials used during recovery (downstream processing), followed by a description of the different recovery process steps:

- Pre-treatment
- Primary solid/ liquid separation
- Concentration
- Polish and germ filtration

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

A.5.8.Materials

Materials used, if necessary, during recovery of the food enzyme include:

- Flocculants
- Filter aids
- pH adjustment agents

Potable water can also be used in addition to the above-mentioned materials during recovery.

A.5.9.Pre-Treatment

Flocculants and/or filter aids are added to the fermentation broth, in order to get clear filtrates, and to facilitate the primary solid/liquid separation.

A.5.10.Primary solid/liquid separation

The purpose of the primary separation is to remove the solids from the enzyme containing fermentation medium. The primary separation is performed at defined pH and temperature ranges in order to minimize loss of enzyme activity.

The separation process may vary, depending on the specific enzyme production plant. This can be achieved by different operations like centrifugation or filtration.

A.5.11.Concentration

The liquid containing the enzyme protein needs to be concentrated in order to achieve the desired enzyme activity and/or to increase the ratio enzyme activity/TOS before formulation. Temperature and pH are controlled during the concentration step, which is performed until the desired concentration has been obtained.

A.5.12.Polish and germ filtration

After concentration, for removal of residual cells of the production strain and as a general precaution against microbial contamination, filtration on dedicated germ filters is applied at various stages during the recovery process. Pre-filtration (polish filtration) is included if needed to remove insoluble substances and facilitate the germ filtration. The final polish and germ filtration at the end of the recovery process results in a concentrated enzyme solution free of the production strain and insoluble substances.

A.5.13.Formulation and Packaging

Following formulation, the final product is defined as a 'food enzyme preparation.' Food enzymes can be sold as dry or liquid preparations, depending on the final application where the enzyme is intended to be used. For all kinds of food enzyme preparations, the food enzyme is standardized and preserved with food ingredients or food additives which are approved in Australia according to ruling legal provisions.

Lipase enzyme preparation from *T. reesei* RF10625 is sold mainly as a powdered product.

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

A.6. Specification for the purity and identity

The final enzyme product complies with the recommended General Specifications for Enzyme Preparations Used in Food Processing Joint FAO/WHO Expert Committee on Food Additives, Compendium of Food Additive Specifications, FAO Food and Nutrition Paper *(Food and Agriculture Organization of the United Nations 2006)* and the Monograph "Enzyme Preparations" Food Chemicals

Codex (FCC) 10th edition (2016) for food-grade enzymes. Specifications for the food enzyme preparation have been defined as follows:

Analytical data is provided in Appendix #5.

The methods used are provided in Appendix #6.

See **Section A.3** for more information regarding physical properties.

A.7. 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

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 safety of the lipase preparation was confirmed or is under consideration by external expert groups, as follows:

- **France**: The enzyme preparation was safety assessed according to the Guidelines for the evaluation of food enzymes. This resulted in the authorisation of the enzyme product by the French authorities in April 2017 (Appendix #7).
- **USA**: A GRAS no objection letter determined that the xylanase enzyme reparation is GRAS for its intended use GRAS #631 (Appendix #8).
- **EFSA/ EU Commission**: A dossier was submitted in 2015 in compliance with Regulation (EC) 1332/2008 and is currently being reviewed by EFSA.

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

As documented below, lipase from various micro-organisms (including genetically modified ones) are widely accepted for their use in several applications. See accordant table below:

Non-exhaustive list of authorised lipases from production organisms other than *Trichoderma reesei*		
Authority	**Production organism**	**Reference**
Canada	*Aspergillus oryzae,* *Rhizopus oryzae var* *Rhizomucor miehei* *Rhizopus niveus* *Candida cylindracea* *Penicillium camembertii* ***Lipase from animal origin:*** *- Animal pancreatic tissue* *- Edible forestomach of calves, kids of lambs*	https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/5-enzymes.html
Australia/ New Zealand **[Schedule 18, Standard 1.3.3]**	*Aspergillus niger* *Aspergillus niger,* containing the gene for lipase, triacylglycerol isolated from *Fusarium culmorum* *Aspergillus oryzae* *Aspergillus oryzae,* containing the gene for Lipase, triacylglycerol isolated from *Fusarium oxysporum* *Aspergillus oryzae,* containing the gene for Lipase, triacylglycerol isolated from *Humicola lanuginose* *Aspergillus oryzae,* containing the gene for Lipase, triacylglycerol isolated from *Rhizomucor miehei* *Candida rugose* *Hansenula polymorpha,* containing the gene for Lipase, triacylglycerol isolated from *Fusarium heterosporum*	https://www.legislation.gov.au/Details/F2016C00166 FSANZ, Application A516 FSANZ, Application A435

	Mucor javanicus *Penicillium camenbertii* *Penicillium roquefortii* *Rhizopus arrhizus* *Rhizomucor miehei* *Rhizophus niveus* *Rhizophus oryzae* ***Lipase from animal origin:*** *- Bovine stomach* *- Salivary glandof forestomach of calf* *-porcine or bovine pancreas*	
France	*Aspergillus oryzae,* containing the gene for Lipase, triacylglycerol isolated from *Fusarium oxysporum* *Aspergillus oryzae,* containing the gene for Lipase, triacylglycerol isolated from *Thermomyces lanuginosus /Fusarium oxysporum* *Aspergillus oryzae,* containing the gene for Lipase, triacylglycerol isolated from *Humicola lanuginosa* *Candida rugose* *Aspergillus niger,* containing the gene for Lipase, triacylglycerol isolated from *Thermomyces lanuginosus* *Aspergillus niger,* containing the gene for Lipase, triacylglycerol isolated from *Fusarium culmorum* *Pichia angusta,* containing the gene for Lipase, triacylglycerol isolated from *Fusarium heterosporum* *Rhizopus oryzae* *Rhizopus niveus*	Arrêté du 19 octobre 2006 relatif à l'emploi d'auxiliaires technologiques dans la fabrication de certaines denrées alimentaires | Legifrance
USA	*Aspergillus oyzae,* containing the gene for Lipase, triacylglycerol isolated from *Thermomyces lanuginosus* *Penicillium camenmbertii* *Aspergillus oryzae,* containing the gene	GRAS Notice GRN043 GRAS Notice GRN068 GRAS Notice GRN 075 GRAS Notice GRN 081

	for Lipase, triacylglycerol isolated from *Fusarium oxysporum* *Candida rugose* *Aspergillus oryzae* carrying a gene constructed from a modified *Thermomyces lanuginosus* lipase gene and a portion of the *Fusarium oxysporum* lipase gene *Aspergillus niger* *Aspergillus oryzae* *Aspergillus niger* expressing a gene encoding a lipase from *Candida antartica* *Rhizopus oryzae* *Hansenula polymorpha* expressing a gene encoding a lipase from *Fusarium heterosporum* *Pseudomonas fluorescens* Biovar I	GRAS Notice GRN 103 GRAS Notice GRN 111 GRAS Notice GRN 296 GRAS Notice GRN 113 GRAS Notice GRN 158 GRAS Notice GRN 216 GRAS Notice GRN 238 GRAS Notice GRN 462

The enzyme preparation from *Trichoderma reesei* produced triacylglycerol expressing a gene from *Fusarium oxysporum* was evaluated according to the Pariza and Johnson Decision Tree. The decision tree is based on the safety evaluation published by Pariza and Foster in 2001, adapted from their original evaluation in 1983. Based on the Pariza and Johnson decision tree analysis, AB Enzymes concludes that the lipase enzyme preparation is safe, see Appendix #9.

For information regarding sales globally, please refer to Appendix #13-CCI.

C.2.2.Toxicological Studies

This section describes the studies performed to evaluate the safety of the RF10625 lipase enzyme preparation. All safety studies were performed according to internationally accepted guidelines (OECD or FDA) and are in compliance with the principles of Good Laboratory Practice (GLP) according to the FDA/OECD.

It is generally accepted that known commercial enzyme preparations of *T. reesei* are not toxic and since lipase is a natural constituent in the environment, it is concluded that the lipase enzyme from *T. reesei* RF10625 is safe as for use as a food processing aid in various applications.

To further confirm that the lipase enzyme preparation does not have any toxic properties and to ensure the toxicological safety of the use of the enzyme preparation from *T. reesei*, the following studies were conducted:

- Ames test – Appendix #10
- Chromosomal aberration test, in vitro – Appendix #11
- 90 Day Oral Toxicity Study (Rodents) – Appendix #12

C.2.2.1. Bacterial Reverse Mutation Test

The test, based on OECD Guidelines No. 471 was run at Harlan, Cytotest Cell Research GmbH (Harlan CCR) Rossdorf – Germany. The study was completed on August 15, 2013.

This study was performed to investigate the potential of lipase from *Trichoderma reesei* RF10625 to induce gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and TA 102.

The assay was performed in two independent experiments both with and without liver microsomal activation. To verify a minor increase in strain TA 98 without S9 mix, confirmatory experiments IIa and IIb were performed with strain TA 98 without S9 mix as pre-incubation assay. Each concentration, including the controls, was tested in triplicate.

The test item was tested at the following concentrations:

— Pre-Experiment/Experiment I: 3; 10; 33; 100; 333; 1,000; 2,500; and 5,000 µg/plate

— Experiment II: 33; 100; 333; 1,000; 2,500; and 5,000 µg/plate

— Experiment IIa and II b without filtration: 2,500; 5,000 and 10,000 µg/plate

— Experiment IIb with filtration: 2,500 and 5,000 µg/plate

No precipitation of the test item occurred up to the highest investigated dose.
The plates incubated with the test item showed normal background growth up to 5,000 µg/plate with and without S9 mix in all strains used.

No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5) occurred in the test groups with and without metabolic activation. Only strain TA102 showed a minor reduction in the number of revertants in Experiment II with S9 mix at 5,000 µg/plate.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with lipase at any dose level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.

Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

Therefore, the lipase from *Trichoderma reesei* RF10625 was considered to be non-mutagenic in this *Salmonella typhimurium* reverse mutation assay.

C.2.2.2. Chromosomal Aberration Test

The test, based on OECD Guidelines No. 473 was run at Harlan, Cytotest Cell Research GmbH (Harlan CCR) Rossdorf – Germany. The study was completed on September 02, 2013.

The lipase from *Trichoderma reesei* RF10625 was assessed for its potential to induce structural chromosome aberrations in human lymphocytes *in vitro* in two independent experiments. The following study design was performed:

	Without S9 mix		With S9 mix
	Experiment I	Experiment II	Experiment III
Exposure period	4 hrs	22 hrs	4 hrs
Recovery	18 hrs	-	18 hrs
Preparation interval	22 hrs	22 hrs	22 hrs

In each experimental group, two parallel cultures were analysed. Per culture 100 metaphases were evaluated for structural chromosome aberrations.

The highest applied concentration (5,300 µg/mL = 5,000 µg/mL adjusted to TOS) was chosen with respect to the current OECD Guideline 473. Dose selection for the cytogenetic experiments was performed considering the toxicity data in accordance with OECD GL 473.

In both cytogenetic experiments, in the absence and presence of S9 mix, at the highest evaluated concentrations the mitotic indices were clearly reduced. Either with or without metabolic activation, no clastogenicity was observed at the concentrations evaluated. However, in the presence of S9 mix, one increase in chromosomal aberrations (3.3 % aberrant cells, excluding gaps) slightly above the laboratory historical solvent control data (0.0 – 3.0 % aberrant cells, excluding gaps) was observed after treatment with 322.9 µg /mL. Since the value is not statistically significant this finding has to be regarded as being biologically irrelevant.

No evidence of an increase in polyploid metaphases was noticed after treatment with the test item as compared to the control cultures. Appropriate mutagens were used as positive controls. They induced statistically significant increases in cells with structural chromosome aberrations.

No toxic effects indicated by reduced mitotic indices and/or reduced cell numbers of below 50 % of control were observed after treatment up to the highest required test item concentration.

In both independent experiments, no biologically relevant increase in the number of cells carrying structural chromosomal aberrations was observed after treatment with the test item. However, in Experiment II in the presence of S9 mix a single significant increase (2.0 %) was observed but this value was clearly within the laboratory's historical control data range (0.0 – 4.0 % aberrant cells, excluding gaps) and is regarded as biologically irrelevant.

No evidence of an increase in polyploid metaphases was noticed after treatment with the test item as compared to the control cultures.

Appropriate mutagens were used as positive controls. They induced statistically significant increases in cells with structural chromosome aberrations.

In conclusion, it can be stated that under the experimental conditions reported, the test item did not induce structural chromosomal aberrations in human lymphocytes *in vitro*.

Therefore, the lipase from *Trichoderma reesei* RF10625 is considered to be non-clastogenic in this chromosome aberration test, when tested up to cytotoxic concentrations. *In vivo* tests were not performed, as there was no *in vitro* mutagenicity detected.

C.2.2.3. 90-Day Sub-Chronic Toxicity Study

The test was performed according to the following guidelines: OECD No. 408 at Harlan Laboratories Ltd (Itingen, Switzerland). The study was completed on April 30, 2014.

In this subchronic toxicity study, lipase from *Trichoderma reesei* RF10625 was administered daily by oral gavage to SPF-bred Wistar rats of both sexes at dose levels of 50, 200 and 1,000 mg/kg body

weight/day for a period of 92/93 days. A control group was treated similarly with the vehicle, bi-distilled water, only.

The groups comprised 10 animals per sex which were sacrificed after 92/93 days of treatment. Clinical signs, outside cage observation, food consumption and body weights were recorded periodically during the acclimatization, treatment and recovery periods. Functional observational battery, locomotor activity and grip strength were performed during week 13.

At the end of the dosing and the treatment-free recovery period, blood samples were withdrawn for hematology and plasma chemistry analyses. Urine samples were collected for urinalyses. All animals were killed, necropsied and examined post mortem. Histological examinations were performed on organs and tissues from all control and high dose animals, and all gross lesions from all animals.

- Mortality / Viability: There was not test item-related mortality.
- Clinical Signs (Daily and Weekly): There were no test item-related findings in the daily or weekly observations.
- Functional Observational Battery: There were no test item-related findings in the functional observation battery at week 13.
- Grip Strength and Locomotor Activity: Grip strength and locomotor activity of test item-treated rats were unaffected.
- Food Consumption: There were no test item-related effects
- Body Weights: There were no test item-related effects.
- Ophthalmoscopic Examinations: There were no test item-related effects.
- Hematology/ Clinical Biochemistry / Urinalysis: There were no changes of toxicological relevance.
- Organ Weights: There were no test item-related effects.
- Macroscopic / Microscopic Findings: There were no unscheduled deaths.

Conclusion: Based on the results of this study, 1,000 mg/kg body weight/day of Lipase produced with *Trichoderma reesei* RF10625 was established as the no-observed-effect-level (NOEL) and 1,000 mg/kg body weight/day as the no-observed-adverse-effect-level (NOAEL).

C.3. Information on any Significant Similarity between the Amino Acid Sequence of the Enzyme and that of Known Protein Toxins.

A homology search was performed from the non-redundant protein sequences database using the BLAST-P (protein – protein BLAST) program, v. 2.6.1+ (http://blast.ncbi.nlm.nih.gov/). The amino acid sequence of the lipase (Appendix #13 – treated as confidential) was used as the query sequence in the searches.

BLAST-P is a basic local alignment search tool. By using this tool identities between two protein sequences can be found if the proteins contain similar sequence stretches (domains) even though the overall sequence homology between the sequences might be very low.

According to the results obtained from the searches performed it can be concluded that the lipase protein does not shown significant homology to any protein sequence identified or known to be a toxin.

C.4. Information on the Potential Allergenicity of the Enzyme Processing Aid

C.4.1. The source of the Enzyme Processing Aid

The dossier concerns a triacylglycerol lipase gene from *Fusarium oxysporum* expressed in *T.reesei*.

Name of the enzyme protein: **Triacylglycerol lipase**

Production strain: *Trichoderma reesei* RF10625

C.4.2.Donor

The *Fusarium oxysporum* lipase gene for lipase protein overproduced by RF10625 was designed and chemically synthesized using the preferred codon usage for *Trichoderma reesei*. A codon-optimized *Fusarium oxysporum* lipase encoding sequence was designed based on the *Hypocrea jecorina* preferred codon usage (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=51453) and synthesized by Eurofins (Germany).

C.4.3. An Analysis of Similarity between the Amino Acid Sequence of the Enzyme and that of known Allergens.

As some enzymes manufactured for use in food have been reported to cause inhalation allergy in workers exposed to enzyme dust in manufacturing facilities, lipase may also cause such occupational allergy in sensitive individuals. However, the possibility of an allergic reaction to the lipase residues in food seems remote. In order to address allergenicity by ingestion, it may be taken into account that:

- The allergenic potential of enzymes was studied by *Bindslev-Jensen et al. (2006)* and reported in the publication: "*Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry*". The investigation comprised enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and protein engineered variants and comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. It was concluded from this study that ingestion of food enzymes in general is not likely to be a concern with regard to food allergy.
- Previously, the AMFEP Working Group on Consumer Allergy Risk from Enzyme Residues in Food performed an in-depth analysis of the allergenicity of enzyme products (Daurvin et al. 1998). The overall conclusion is that exposure to enzyme proteins by ingestion, as opposed to exposure by inhalation, are not potent allergens and that sensitization to ingested enzymes is rare.

Thus, there are no scientific indications that small amounts of enzymes in food can sensitize or induce allergic reactions in consumers.

Additional considerations supporting the assumptions that the ingestion of an enzyme protein is not a concern for food allergy should also be taken into account:

- The majority of proteins are not food allergens and based on previous experience, the enzyme industry is not aware of any enzyme proteins used in food that are homologous to known food allergens.
- The food enzyme is used in small amounts during food processing, resulting in very small amounts of the enzyme protein in the final food. A high concentration generally equals a higher

risk of sensitization, whereas a low level in the final food equals a lower risk (Goodman et al. 2008).

- In the case where proteins are denatured - which is the case for this lipase - due to the food process conditions (i.e starch process), the tertiary conformation of the enzyme molecule is destroyed. In general, these alterations in conformation are associated with decrease in the antigenic reactivity in humans: in the vast majority of investigated cases, denatured proteins are much less immunogenic than the corresponding native proteins (Valenta, Kraft 2002; Valenta 2002; Takai et al. 1997; Takai et al. 2000; Nakazawa et al. 2005; Kikuchi et al. 2006)
- In addition, residual enzyme still present in the final food will be subjected to digestion in the gastro-intestinal system, which reduces further the risk of enzyme allergenicity. While stability to digestion is considered as a potential risk factor of allergenicity, it is believed that small protein fragments resulting from digestion are less likely to be allergenic
- Finally, enzymes have a long history of safe use in food processing, with no indication of adverse effects or reactions. Moreover, a wide variety of enzyme classes (and structures) are naturally present in food. This is in contrast with most known food allergens, which are naturally present in a narrow range of foods.

In order to specifically evaluate the risk that lipase enzyme would cross react with known allergens and induce a reaction in an already sensitized individual, sequence homology testing to known allergens was performed.

A sequence homology comparison test was then performed using a database of allergens from the Food Allergy Research and Resource Program (FARRP), University of Nebraska, Allergen Database (Version 14, January 20, 2014), which contains the amino acid sequences of known and putative allergenic proteins (Appendix #13 – treated as CCI.)

The resulting alignments of the full-length lipase protein sequence to any allergenic proteins in the allergen database showed no sequences with E () <1.000000. In addition, the lipase protein sequence

showed no matches of greater than 35% to the known allergens when searching for 80 amino acid alignments and no perfect match when searching for a stretch of eight amino acids.

Based on the results obtained from the bioinformatics approach to estimate potential allergenicity on relatedness to known allergens and taking into account the most recent scientific recommendations on the interpretation of such data, and based on the fact that the enzyme is typically denatured during the food manufacturing process and that any residual enzyme still present in the final food will be subject to digestion in the gastro-intestinal system, it is unlikely that the lipase produced by *Trichoderma reesei* RF10625 under evaluation will cause allergic reactions after ingestion of food containing the residues of these enzymes.

Conclusion:

Based on the results obtained from the bioinformatics approach to estimate potential allergenicity on relatedness to known allergens and taking into account the most recent scientific recommendations on the interpretation of such data, and based on the fact that the enzyme is typically denatured during the food manufacturing process and that any residual enzyme still present in the final food will be subject to digestion in the gastro-intestinal system, it is not likely that the lipase produced by *Trichoderma reesei* RF10625 under evaluation will cause allergic reactions after ingestion of food containing the residues of these enzymes

C.5. Safety assessment reports prepared by international agencies or other national government agencies, if available

Please see section C.1.

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

D.1. Information on the source organism

The microorganism that is used for the production of lipase is the fungus *Trichoderma reesei*.

Scientific name:

Genus:Trichoderma

Species: *Trichoderma reesei*

Taxonomy: *Trichoderma reesei* is a hypercellulolytic fungus which was found on deteriorating military fabrics such as tents and clothing. This isolate, designated as QM6a, was initially named *Trichoderma viride*. Approximately 20 years later, QM6a was re-classified as *Trichoderma reesei*. In the 1980s, it was suggested that *Trichoderma reesei* should be placed into synonymy with *Trichoderma longibrachiatum* (Bissett 1991). Later however, evidence appeared that the two species were not identical (Meyer et al. 1992) and it was decided to go back to the *Trichoderma reesei* name. It is of relevance to note that enzymes have been approved that are produced by *T. reesei* under the name of *T. longibrachiatum*. Taxonomic studies have shown that the species *Trichoderma reesei* consists only of this single isolate QM6a and its derivatives (e.g. Rut Series, Montenecourt and Eveleigh, 1977, 1979; QM9123 and QM9414, Mandels et al, 1971 – as reviewed by Nevalainen et al. (1994)). The American Type Culture Collection (ATCC) designation for this original strain of *Trichoderma reesei* QM6a is ATCC 13631.

Synonyms[3]**:** T*richoderma reesei* is the species name given to the anamorphic form (the form which reproduces asexually) of the fungus whose telemorphic form (the form which reproduces sexually) is now understood to be *Hypocrea jecorina* (Kuhls et al. 1996; Seidl et al. 2008). *Trichoderma reesei* was formerly known as *Trichoderma longibrachiatum*.

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

Species belonging to the genus *Trichoderma* are common in soil as well as on vegetable debris and they are widespread all over the world. *Trichoderma reesei* strains have been isolated from soil (compost material) only at low altitudes and within a narrow belt around the equator (± 20 degrees altitude; (Kubicek et al. 2008). The original isolate, QM6a (MANDELS, REESE 1957) was isolated from the

[3] Reference: Mycobank taxonomic database - Search Term "Trichoderma reesei" (see: http://www.mycobank.org/Biolomics.aspx?Table=Mycobank&Page=200&ViewMode=Basic).

Solomon Islands in 1944. As *T. reesei is a goo*d producer of cellulases, it has been widely studied in several laboratories and developed as industrial enzyme producer using random mutagenesis and genetic engineering. The original isolate, QM6a is the initial parent of practically all currently industrially relevant food enzyme production strains, including our strain RF10625.

Trichoderma reesei has a long history (more than 30 years) of safe use in industrial-scale enzyme production (Nevalainen et al. 1994; Blumenthal 2004). E.g. cellulases, hemicellulases, β-glucanases, pectinases and xylanases produced by this fungus are used in food, animal feed, pharmaceutical, textile, detergent, bioethanol and pulp and paper industries.

Food enzymes derived *Trichoderma reesei* strains (including recombinant *T. reesei* strains) have been evaluated by JECFA and many countries which regulate the use of food enzymes, such as the USA, France, Denmark, Australia and Canada, resulting in the approval of the use of food enzymes from *Trichoderma reesei* in the production of various foods, such as baking, brewing, juice production, wine production and the production dairy products.

Pathogenicity:

Trichoderma reesei strains are non-pathogenic for healthy humans and animals (Nevalainen et al. 1994).

Trichoderma reesei is not listed in Annex III of Directive 2000/54/EC – which lists microorganisms for which safety concerns for workers exist-as it is globally regarded as a safe microorganism:

- In the USA, *Trichoderma reesei* is not listed as a Class 2 or higher Containment Agent under the National Institute of Health Guidelines for Recombinant DNA Molecules. Data submitted in Generally Recognized as Safe (GRAS) petitions to the Food and Drug Administration (FDA) for numerous enzyme preparations from T. *reesei* for human and animal consumption demonstrate that the enzymes are nontoxic. The Environmental Protection Institute (EPA) completed a risk assessment on *T. reesei* in 2011 resulting in a Proposed Rule in 2012, concluding that it is appropriate to consider *T. reesei* as a recipient microorganism eligible for exemptions from full reporting requirements[4], if

[4] reporting procedures in place under the Toxic Substances Control Act (TSCA) for new micro-organisms that are being manufactured for introduction into the commerce. https://www.gpo.gov/fdsys/pkg/FR-2012-09-05/html/2012-21843.htm

this fungus was to be used in submerged standard industrial fermentation for enzyme production.

- In Europe, *Trichoderma reesei* is classified as a low-risk-class microorganism, as exemplified by being listed as Risk Group 1 in the microorganism classification lists of the German Federal Institute for Occupational Safety and Health (BAuA[5]) and the Federal Office of Consumer Protection and Food Safety (BVL), and not appearing on the list of pathogens from Belgium (Belgian Biosafety Server, 2010[6]).

As a result, *Trichoderma reesei* can be used under the lowest containment level at large scale, GILSP, as defined by OECD (ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT 1992).

Secondary metabolites in *Trichoderma reesei* (*Hypocrea jecorina*) strains:

The safety of *Trichoderma reesei* has been discussed in several review papers (Nevalainen et al. 1994; Blumenthal 2004; Kubicek et al. 2011; Peterson, Nevalainen 2012). *T. reesei* has been described not to produce mycotoxins or antibiotics under conditions used for enzyme production.

It is recognized that *Trichoderma reesei* is capable of producing peptaibols (e.g. paracelcin) and that the *Trichoderma reesei* genome contain genes for two peptaibol synthases (Kubicek et al. 2011). However, the bulk of the literature investigating the capability of *Trichoderma reesei* to produce peptaibols is based on fermentation conditions designed either to mimic natural (and stressful) growth conditions or attempt to optimize the conditions for secondary metabolite production. These methods are not representative of the conditions used in controlled industrial fermentation practices:

- Under controlled industrial fermentation conditions, the organisms are not subjected to significant stress: the literature indicates that the biosynthesis of peptaibols is a defence response against other fungi when subjected to environmental stress such as the lack of nutrients (Tisch, Schmoll 2010; Komon-Zelazowska et al. 2007).
- Standard industrial fermentation process times are short for peptaibols induction: peptaibols have mostly been isolated from very old cultures of *Trichoderma*, at least 15

[5] http://www.bvl.bund.de/SharedDocs/Downloads/06_Gentechnik/register_datenbanken/organismenliste_2010.pdf?__blob=publicationFile&v=6

[6] https://www.biosafety.be/content/tools-belgian-classification-micro-organisms-based-their-biological-risks

days of cultivation (Kubicek et al. 2007). Industrial fermentation processes for *Trichoderma reesei* can be up to 10 days, but is typically shorter (3-8 days).

From what is described above, it can be concluded that the production of peptaibols by *Trichoderma reesei* strains under controlled and optimized industrial fermentation conditions is of insignificant concern.

It is relevant to note that during recent years, genetic engineering techniques have extensively been used to improve the industrial production strains of *T. reesei*, and in addition, considerable experience of safe use of recombinant *T. reesei* strains in industrial scale has accumulated. Furthermore, food enzymes from *Trichoderma reesei* have been subjected to several testings as part of their safety assessment for the use in food products manufacturing processes including 90-day toxicological tests.

T. reesei RF10625 enzyme fermentation extracts have been subjected to several tests as part of their safety assessment for the production of food products. In toxicological tests that have been performed, including a 90-day repeated dose study, no toxicity of lipase fermentation product as produced by the present production strain *Trichoderma* reesei RF10625 was detected (see **Section C**). These results show that there is no need for any toxicological concern with fermentation products as produced by use of *Trichoderma reesei*.

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

The genetic stability of the strain over the fermentation time was analyzed by southern blotting and no instability of the strain was detected. For more detailed description of the strain construction and characteristics, please see **Section E** below.

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 summarized information. The detailed information is provided in the Appendix #13 – treated as CCI.

E.2. Host/recipient organism

The *T. reesei* recipient is a classical mutant strain originating from *T. reesei* QM6a. The identification of the strain as *T. reesei* has been confirmed by the Centraalbureau voor Schimmelcultures (CBS) in the Netherlands.

E.3. Donor

The *Trichoderma reesei* host strain is genetically modified with a lipase gene deriving from *Fusarium oxysporum*.

E.4. Genetic modification

Trichoderma reesei strain RF10625 was constructed for production of *Fusarium oxysporum* lipase, by transformation of a *T. reesei* host mutant strain with a purified DNA fragment isolated from a plasmid consisting of the fungal expression cassette and a pUC19 vector backbone.

The *Fusarium oxysporum* lipase encoding sequence was designed and synthesized using the preferred codon usage for *Trichoderma reesei*. The plasmid was characterized by restriction with endonucleases and the construct was confirmed by DNA sequencing.

The plasmid was digested with *Not*I and the expression cassette containing the lipase gene was isolated. The purified expression cassette devoid of pUC19 elements was used for transformation of the *T. reesei* mutant host strain.

The transformation of the *T. reesei* mutant host strain with the lipase expression cassette was performed as described in Penttilä et al. (1987) with the modifications described in *Karhunen et al. (1993)*. The transformants were selected according to their ability to grow on acetamide plates. The expression cassettes are integrated into the host genome as several copies.

The expression cassette consists of a *T. reesei* signal sequence and a carrier polypeptide encoding sequences, the lipase gene from *Fusarium oxysporum* and *Aspergillus nidulans amdS* gene sequence (as a selection marker).

According to Southern blot analysis multiple copies of the lipase expression cassette are present in the genome of RF10625.

The DNA fragments that have been transformed into the *T. reesei* mutant host strain are well characterized, the sequences of the genes are known, and the fragments are free of any harmful sequences.

E.5. Stability of the transformed genetic sequence

T. reesei strains are widely used in biotechnological processes because of their known stability. The inserted DNA does not include any mobile genetic elements. Additionally, it should be highlighted that *T. reesei* genome lacks a significant repetitive DNA component and no extant functional transposable elements have been found in the genome (Kubicek et al. 2011; Martinez et al. 2008). This results to low risk of transfer of genetic material.

The stability and potential for transfer of genetic material was assessed as a component of the safety evaluation of the production microorganism. Southern blot analyses were performed to the genome of the *T. reesei* production strain RF10625. Results indicated that several copies of the expression cassettes were integrated in the genome of strain RF10625 and that the production strain is stable in terms of genetic traits.

For more details, please see Appendix #13 – treated as CCI.

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

Triacylglycerol lipase can be used in the manufacturing of cereal based products such as, but not limited to, bread, biscuits, steamed bread, cakes, pancakes, tortillas, wafers and waffles.

Like any other enzyme, lipase acts as a biocatalyst: with the help of the enzyme, a certain substrate is converted into a certain reaction product or products. It is not the food enzyme itself, but the result of this conversion that determines the effect in the food or food ingredient. After the conversion has taken place, the enzyme no longer performs a technological function.

Commercial food enzyme preparations are generally used following the *Quantum Satis* (QS) principle, i.e. at a level not higher than the necessary dosage to achieve the desired enzymatic reaction – according to Good Manufacturing Practice. The amount of enzyme activity added to the raw material by the individual food manufacturer has to be determined case by case, based on the desired effect and process conditions. Therefore, the enzyme manufacturer can only issue a recommended enzyme dosage range. Such a dosage range is the starting point for the individual food producer to fine-tune his process and determine the amount of enzyme that will provide the desired effect and nothing more. Consequently, from a technological point of view, there are no 'normal or maximal use levels' and lipase from *T. reesei* RF10625 is used according to the QS principle. A food producer who would add much higher doses than the needed ones would experience untenable costs as well as negative technological consequences.

Microbial food enzymes contain – apart from the enzyme protein in question – also some substances derived from the producing micro-organism and the fermentation medium. The presence of all organic materials is expressed as Total Organic Solids[7] (TOS, FAO/WHO, 2006). Whereas the dosage of a food enzyme depends on the enzyme activity present in the final food enzyme preparation, the dosage on basis of TOS is more relevant from a safety point of view. Therefore, the use levels are expressed in TOS. The Table below shows the range of recommended use levels for each application where the lipase is to be used.

[7] In the case of food enzymes, which are - per legal definition - not formulated, TOS is the same as Dry Matter minus ash. The amount of ash (e.g. mineral salts used in the fermentation) does generally not exceed a few percent.

Foods Uses for Lipase	
Food Grouping	**Proposed Food Uses**
Cereal-based products and dishes	Used in the manufacturing of bakery products such as, but not limited to, bread, biscuits, steamed bread, cakes, pancakes, tortillas, wafers and waffles. Pastas, noodles

Application and Raw Material	Raw Material	Maximal recommended use levels (mg TOS/kg RM)
Baking and other cereal based processes	Flour	10

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

The most appropriate way to estimate the human consumption in the case of food enzymes is using the so-called Budget Method, originally known as the Danish Budget Method *(Douglass et al. 1997; Hansen 1966)*. This method enables one to calculate a Theoretical Maximum Daily Intake (TMDI) based on conservative assumptions regarding physiological requirements for energy from food and the energy density of food rather than on food consumption survey data.

The Budget Method was originally developed for determining food additive use limits and is known to result in conservative estimations of the daily intake.

The Budget Method is based on the following assumed consumption of important foodstuffs and beverages (for less important foodstuffs, e.g. snacks, lower consumption levels are assumed):

Consumption of food patterns:

Average consumption over the course of a lifetime/kg body weight/day	Total solid food (kg)	Total non-milk beverages (l)	Processed food (50% of total solid food) (kg)	Soft drinks (25% of total beverages) (l)
	0.025	*0.1*	*0.0125*	*0.025*

The recommended use levels of lipase are given based on the raw materials used in the food processes. For the calculation of the TMDI, the maximum use levels are chosen. Furthermore, the calculation takes into account how much food (or beverage) is obtained per kg raw material and it is assumed that all the TOS will end up in the final product and the wide variety of food products that are available to consumers

Applications		Raw material (RM)	Recommended use level (mg TOS/kg RM)	Final food (FF)	Ratio RM/FF*	Maximal level in final food (mg TOS/kg food)
Liquid foods	Baking	Flour	10	No liquid foods	-	-
Solid foods	Baking	Flour	10	Baked products, Pastas and noodles…	0.71	7.1

* Assumptions behind ratios of raw material to final food:

Bakery products fall in the category of solid foods.

Flour is the raw material for bakery product and the yield will vary depending on the type of final food produced.

From 1 kg of flour you would have 4 kg of cakes, 1.4 kg of bread or 1.1 kg of cracker. Cracker may represent the most conservative input from the bakery processes. However, consumption of

bread is higher than that of cracker, this is why bread is used as the assumption for the calculation of dietary exposure from bakery processes.

The yield of 1.4 kg of bread per 1 kg of flour correspond to a RM/FF ratio of 0.71 kg of flour per kg bakery product is used.

The Total Theoretical Maximum Daily Intake (TMDI) can be calculated on basis of the maximal values found in food and beverage, multiplied by the average consumption of food and beverage/kg body weight/day.

The Total TMDI will consequently be: TMDI in food (mg TOS/kg body weight/day)	TMDI in beverage (mg TOS/kg body weight/day)	Total TMDI (mg TOS/kg body weight/day)
7.1 x 0.0125 = 0.09	n/a	**0.09**

It should be stressed that this Total TMDI is based on conservative assumptions and represents a highly exaggerated value because of the following reasons:

- It is assumed that ALL producers of the above-mentioned foodstuffs use the specific enzyme lipase from *Trichoderma reesei*;
- It is assumed that ALL producers apply the HIGHEST use level per application; For the calculation of the TMDI's in food, only THOSE foodstuffs were selected containing the highest theoretical amount of TOS. Thus, foodstuffs containing lower theoretical amounts were not taken into account;
- It is assumed that the amount of TOS does not decrease as a result of the food production process;
- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed DAILY over the course of a lifetime;
- Assumptions regarding food and beverage intake of the general population are overestimates of the actual average levels (Douglass et al. 1997).

Summarizing the results obtained from the several toxicity studies the following conclusions can be drawn:

- No mutagenic or clastogenic activity under the given test conditions were observed;
- The sub-chronic oral toxicity study showed a No Observed Adverse Effect Level (NOAEL) of at least 1000 mg TOS/kg body weight/day.

The Margin of Safety (MoS) for human consumption can be calculated by dividing the NOAEL by the Total Theoretical Maximal Daily Intake (TMDI). Total TMDI of the food enzyme is 0.09 mg TOS/kg body weight/day. Consequently, the MoS is:

- MoS = 1,000 / 0.09 = **11,111**

As is explained above, the Total TMDI is highly exaggerated. Moreover, the NOAEL was based on the highest dose administered, and is therefore to be considered as a minimum value. Therefore, the actual MoS in practice will be some magnitudes higher.

The overall conclusion is that the use of the food lipase *from Trichoderma reesei* RF10625 in the production of food is absolutely safe. Considering the high safety factor – even when calculated by means of an overestimation of the intake via the Budget method – there is no need to restrict the use of the enzyme in food processing.

Consequently, it is concluded that lipase from *Trichoderma reesei* RF10625 can be used *Quantum Satis* in bakery products.

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

Since we used the Budget Method to quantity the potential of residues in the final food consumed by individuals, it is assumed that all products containing the substrate are produced using the lipase enzyme as a processing aid at the recommended dose.

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

The Budget Method assumes a worst-case scenario, and as such it is predicted that all countries would have the same level of residues in the processed food product.

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

Not applicable.

VI. List of appendices

Section 3.1

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Section 3.3

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