AB Enzymes GmbH – Feldbergstrasse 78 , D-6412 Darmstadt

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

AB Enzymes GmbH

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II. EXECUTIVE SUMMARY

The present application seeks to amend table to subsection S18—9(3), Permitted processing aids of various purposes of the Australia New Zealand Food Standards Code (the Code) to approve a lysophospholipase enzyme preparation from *Trichoderma reesei* produced by AB Enzymes GmbH.

Proposed change to Standard 1.3.3 - Processing Aids

The table to 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 lysophospholipase (EC 3.1.1.5) for use in starch processing.

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 lysophospholipase (IUBM 3.1.1.5). The food enzyme catalyzes the hydrolysis of an ester bond between a fatty acid and glycerol in lysophospholipids, resulting in the formation of free fatty acids and glycero-phosphatide.

It uses lysophospholipids as substrates. Lysophospholipids are small (glycerol) phospholipids molecules, formed during the phospholipids breakdown as a result of the action of phospholipases. Although phospholipids are major component of all cell membranes in animals, plants and micro-organisms, lysophospholipids are found in only small amount in biological membranes. However, lysophospholipds and their receptors have been found in a wide range of tissues and cell types, indicating their

importance in many physiological processes. Lysophospholipids are also known to be the predominant phospholipids found in wheat starch. Consequently, the substrate for lysophospholipase occurs naturally in nature (in particular in wheat based foods) and is therefore a natural part of the human diet.

Apart from lysophospholipase, the food enzyme also contains other enzymatic side activities in small amounts, which are naturally and typically produced by the production organism *Trichoderma reesei*, mainly xylanase, beta-glucanase and cellulase. However, these activities are not relevant from an application and/or safety point of view, due to small amounts and the fact that such enzyme activities have been used and approved for decades in food processing.

The production organism is removed during filtration and is not present in the final enzyme preparation.

Use of the Enzyme

In principle, the enzymatic conversion of conversion of lysophospholipids with the help of lysophospholipase can be used in the processing of all food raw materials which naturally contain lysophospholipids.

The food enzyme object of the dossier is typically used in starch processing, ie. such as the production of all kind of syrups (derived from wheat and corn/maize starches mainly).

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

This dossier is specifically submitted for the use of lysophospholipase **in starch processing, i.e. in the production of all kind of syrups** produced from starch, mainly wheat and maize/corn starches.

Depending on the production process and the type of syrups to be produced, different enzymes are used (e.g. amylase, pullulanase…) to degrade starch.

Lysophospholipds present in starch (mainly wheat starch) can form micelles which negatively affect the filtration rate of the starch hydrolysates (syrups). In addition, they are known to form a complex with amylase, leading to a formation of a cloud in the final syrup, thus affecting its characteristics.

Therefore, the benefits of the conversion of lysophospholipids with the help of lysophospholipase are listed below:

- Prevent the formation of lysophospholipid micelles
- Facilitate the separation of undesired components
- Improve filtration rate (better and faster filtration)
- Improve the characteristics (clearness) of the filtrate
- Improve the environmental impact and sustainability (energy saving due to the load mitigation and decreased production time)

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, edition 10, 2016.

The product is free of production strain and recombinant DNA.

The safety of the *LPL* enzyme 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 July of 2013.
- **USA**: A GRAS determination was conducted and notified to the US FDA in May 2016 (GRN000653). In the reply letter from FDA, the agency had no questions regarding AB Enzymes' determination that the *LPL* enzyme reparation is GRAS for its intended use.
- **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 *Trichoderma reesei* expressing a lysophospholipase (*LPL*) gene from *Aspergillus nishimurae (ex A. fumigatus)*[1] in the table to Schedule 18-9(3) standard 1.3.3.; Permitted processing aids for various technological purposes.

[1] The strain was first identified as *Aspergillus fumigatus in 1999* and was recently identified by CBS as *Aspergillus nishimurae* within the section Fumigati of *Aspergillus*. As the name *Aspergillus fumigatus* has been used in our publications on lysophospholipase deriving from this strain, both names *Aspergillus fumigatus* and *Aspergillus nishimurae* are used for the donor organism.

III. INTRODUCTION

The dossier herein describes a *Trichoderma reesei* produced *LPL* (RF7206) expressing a gene from *Aspergillus nishimurae (ex A. fumigatus*) produced by submerged fermentation.

This dossier is specifically submitted for the use of lysophospholipase in starch processing, i.e. in the production of all kind of syrups produced from starch, mainly wheat and maize/corn starches. Depending on the production process and the type of syrups to be produced, different enzymes are used (e.g. amylase, pullulanase) to degrade starch.

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.

Subsequent 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 levels of use for *LPL* are described. Information on the mode of action, applications, and use levels of *LPL* and enzyme residues in final food products are described. The safety studies outlined herein indicate that the *LPL* 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

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 to schedule 18—9(3), Standard 1.3.3., Permitted processing aids various purposes is proposed to be amended to include a genetically modified strain of *Trichoderma reesei* as permitted source for lysophospholipase (EC 3.1.1.5).

3.1.4. Justification for the Application

The need for the proposed change:

Trichoderma reesei expressing a *LPL* gene from *Aspergillus nishimurae (ex A. fumigatus)* is not present as an approved source in the table of schedule 18—9(3), standard 1.3.3.; Permitted processing aids of various purposes. AB Enzymes GmbH is requesting that this source organism be added.

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

In principle, the enzymatic conversion of lysophospholipids with the help of lysophospholipase can be used in the processing of all food raw materials which naturally contain lysophospholipids.

The 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.

Lysophospholipase from AB Enzymes has a higher activity compared to the other competitive products on the market, which means less excipients is added when applying the enzyme preparation. We are aware of increasing pressure on what is added into the food chain and the potential carry over into the final food. The higher activity of the final product allows for a lower enzyme dose rate.

When used in wheat starch processing, we have observed an improvement in the clarity of the syrup prior to clarification. This results in a further increase in filtration rate, longer filtration runs, reduced consumption of diatomaceous earth (when using rotary vacuum filtration and less membrane fouling and cleaning requirements (when using cross-flow filtration).

Due to the effectiveness of this enzyme in the above-mentioned food processes, AB Enzymes has received authorization to sell in both the USA and France. An application has been submitted to the European Food Safety Authority (EFSA) and is 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 Standard 1.3.3, to table of 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 to starch processing. 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 involved in starch processing. 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. Lysophospholipase 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 lysophospholipase 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. 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. 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 for starch processing is not restricted by any Codex Alimentarius Commission (Codex) Standards or any other known regulations.

National Standards:

The use of this enzyme has been granted a No Objection Letter - GRN #653 in the USA.

Use of enzymes as processing aids in food applications (baking, brewing, starch processing, etc.) has specific standard in France (arrêté du 19 octobre 2006[2]), and the use of this enzyme has been approved for the accordant food applications in this dossier (please see **Section C.1**).

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

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.

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

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 lysophospholipase enzyme, produced with the help of *Trichoderma reesei* strain RF7206. The representative current commercial product is Rohalase® F.

Lysophospholipase is a microbial produced enzyme and already belongs to the table 18-4 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 *LPL* used in starch processing. A further description of the enzyme in these food technology application will be given in subsequent sections.

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

A.2.1.Enzyme

Systematic name	2-lysophosphatidylcholine acylhydrolase
Common names	lecithinase B; lysolecithinase; phospholipase B; lysophosphatidase; lecitholipase; phosphatidase B; lysophosphatidylcholine hydrolase; lysophospholipase A1; lysophopholipase L2; lysophospholipase-transacylase; neuropathy target esterase; NTE; NTE-LysoPLA; NTE-lysophospholipase; 2-lysophosphatidylcholine acylhydrolase
Donor	*Aspergillus nishimurae (ex A. fumigatus)*[3]
Host	*Trichoderma reesei*
Enzyme Commission No.	EC 3.1.1.5

[3] The strain was first identified as *Aspergillus fumigatus in 1999* and was recently identified by CBS as *Aspergillus nishimurae* within the section Fumigati of *Aspergillus*. As the name *Aspergillus fumigatus* has been used in our publications on lysophospholipase deriving from this strain, both names *Aspergillus fumigatus* and *Aspergillus nishimurae* are used for the donor organism.

CAS number	9001-85-8

A.2.2.Enzyme Preparation

The commercial names representative of the enzyme preparation, formulated with the enzyme produced with RF7206 *T. reesei*, is Rohalase® F. The product data sheets are provided in Appendix #1.

A.2.3.Enzyme preparation compositions:

Composition Rohalase® F	
Water	31.35%
Glycerol	50%
Lysophospholipase	13.3%
Sodium chloride	5%
Sodium benzoate	0.35%

The main activity of the enzyme preparation is lysophospholipase (IUB 3.1.1.5), which has been identified in many sources, including plants, microorganisms and animals.

Lysophospholipase catalyzes the hydrolysis of an ester bond between a fatty acid and glycerol in lysophospholipids, resulting in the formation of free fatty acids and glycero-phosphatide. The reaction catalyzed can be described as follows:

2-lysophosphatidylcholine + H2O = glycerophosphocholine + a carboxylate

The substrates for lysophospholipase are lysophospholipids.

Phospholipids are major component of all cell membranes in animals, plants and micro-organisms (they

naturally occur in most vegetable oils (e.g. soya, rapeseed, sunflower oils), marine oils, animal fats (e.g. bovine milk), chicken eggs, fish eggs, etc.-). In general, phospholipids are diacylglycerol molecules with the third carbon attached to a phosphate molecule.

Lysophospholipids (*LPLs*) are small (glycerol)phospholipids molecules, characterized by a single carbon chain and a polar head group, in which one of its two 0- acyl chains is lacking and then only one hydroxyl group of the glycerol backbone is acylated. They are formed during the phospholipid breakdown as a result of the action of phospholipases. Unlike phospholipids, *LPLs* are found only in small amounts in biological cell membranes (Birgbauer, Chun 2006) but *LPLs* and their receptors have been found in a wide range of tissues and cell types, indicating their importance in many physiological processes. [Moolenaar, 2000; Torkhovskaya et al, 2007 as reviewed by D'Arrigo, Servi (2010). Lysophospholipids are also known to be the predominant phospholipids found in wheat starch (Morrison, 1988 as mentioned in Matser, Steeneken (1998)).

Consequently, the substrate for lysophospholipase occurs naturally in nature, and in particular in vegetable (wheat) based foods and is therefore a natural part of the human diet.

Reaction products: as a result of the catalytic activity of lysophospholipase low levels of free fatty acids and glycero-phosphatides are formed. These compounds are already present in the human diet.

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

A.2.4.Genetic Modification

The enzyme is from a *Trichoderma reesei* host strain genetically modified with a *LPL* gene deriving from *Aspergillus nishimurae (ex A. fumigatus)*. The enzyme is not protein engineered.

Name of the enzyme protein: **Lysophospholipase**

Donor: *Aspergillus nishimurae*

Host: *Trichoderma reesei*

Production strain: *Trichoderma reesei* RF7206

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 – Rohalase® F

Properties	
pH Value	5-5.4
Density	1.00-1.10 g/ml
Appearance	Light brown 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, the *LPL* 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.

In starch processing, the lysophospholipase exerts its function during the production of starch

hydrolysates, in particular syrups and sweeteners produced from wheat, corn (mainly) barley, potato, rice and sorghum starches, in order to contribute to an improved and consistent starch saccharification process (Słomińska, Niedbach 2009). After saccharification, the syrup is heated to a temperature of 85°C at which all enzyme activity is inactivated. Further purification steps of the syrups such as activated carbon filtration and ion exchange refining will remove most of the inactivated enzyme which just represents a small fraction of protein in the final syrup.

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

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

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

A.4. Manufacturing Process

Like all food enzymes, *LPL* 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 RF7206 *LPL* 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.4.1.Fermentation

The *LPL* 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.4.2.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.4.3.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)

It is important to note that allergens used during fermentation are consumed and do not end up in the final enzyme product.

A.4.4.Inoculum

A suspension of a pure culture of *T. reesei* RF7206 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.4.5.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.4.6.Main fermentation

Biosynthesis of the *LPL* enzyme product by the production strain *T. reesei* RF7206 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.4.7.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.4.8.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. These substances are allowed in food contact.

A.4.9.Pre-Treatment

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

A.4.10.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 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.4.11.Concentration

The liquid containing the enzyme protein needs to be concentrated 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.4.12.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.4.13.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.

Lysophospholipase enzyme preparation from *T. reesei* strain RF7206 is sold mainly as liquid preparations (could be sold as solid as well, depending on the final application where the enzyme is intended to be used). Please see section A.2.3 for formulation ingredients of the final enzyme preparation.

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.5. 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.6. Analytical method for detection

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

B. Information Related to the Safety of a Chemical Processing Aid

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 *LPL* 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 (EFSA GL, 2009). This resulted in the authorisation of the enzyme product by the French authorities. The approval letters from the French authorities and the ANSES scientific opinions are included in Appendix #7.
- **USA**: A GRAS determination was notified to the US FDA in May 2016 (GRN000653 Appendix #8). In the reply letter from FDA, the agency has no questions regarding AB Enzymes' determination that the lysophospholipase enzyme reparation is GRAS for its intended use.
- **EFSA/ EU Commission**: a dossier was submitted in 2015 in compliance with Regulation (EC) 1332/2008 and is currently being reviewed by EFSA.

The commercial product, Rohalase® F has been sold since 2012 in the UK, Europe, India and Colombia.

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, *LPL* 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 authorisations of authorised lysophospholipase from production organisms		
Authority	**Production organism**	**Reference**
Australia/ New Zealand	*Aspergillus niger*	**Standard 1.3.3 processing aids**
USA	*Trichoderma reeesei*	**GRAS #653**
France	*Aspergillus niger* *Aspergillus niger in* *Aspergillus niger*	**Arrêté du 19 octobre 2006 relatif à l'emploi d'auxiliaires technologiques dans la fabrication de certaines denrées alimentaires | Legifrance**

The *LPL* enzyme preparation from *T. reesei* RF7206, expressing the recombinant gene (*LPL*) deriving from *Aspergillus nishimurae (ex A. fumigatus)* 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 *LPL* enzyme preparation is safe, see Appendix #9.

C.2.2.Toxicological Studies

This section describes the studies performed to evaluate the safety of the RF7206 *LPL* 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 *LPL* is a natural constituent in the environment, it is concluded that the *LPL* enzyme from *T. reesei* RF7206 is safe as for use as a food processing aid in various applications.

To further confirm that the *LPL* 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

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 February 14, 2008.

This study was performed to investigate the potential of lysophospholipase from *Trichoderma reesei* RF7206 to induce gene mutations according to the plate incorporation test (experiment I) and the preincubation 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. 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

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 occurred in the test groups with and without metabolic activation.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with lysophospholipase 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 lysophospholipase from *Trichoderma reesei* RF7206 was considered to be non-mutagenic in this Salmonella typhimurium reverse mutation assay.

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 April 25, 2008.

The lysophospholipase from *Trichoderma reesei* RF7206 was assessed for its potential to induce

structural and numerical chromosome aberrations in V79 cells of the Chinese hamster in vitro in two independent experiments.

In each experimental group two parallel cultures were set up. Per culture 100 metaphases were scored for structural chromosome aberrations.

The highest applied concentration (5,441 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.

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 relevant increase in the frequencies of polyploid metaphases was found after treatment with the test item as compared to the frequencies of the controls. Appropriate mutagens were used as positive controls. They induced statistically significant increases ($p < 0.05$) in cells with structural chromosome aberrations.

In conclusion, it can be stated that under the experimental conditions reported, no biologically relevant increases of chromosomal aberrations were observed.

Therefore, the lysophospholipase from *Trichoderma reesei* RF7206 is considered to be non-clastogenic

in this chromosome aberration test with and without S9 mix when tested up to the highest concentration required by the guideline and adjusted to TOS.

In vivo tests were not performed, as there was no in vitro mutagenicity detected.

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 May 29, 2009.

In this subacute toxicity study, lysophospholipase from *Trichoderma reesei* RF7206 was administered daily by oral gavage to SPF-bred Wistar rats of both sexes at dose levels of 100, 300 and 1,000 mg/kg body weight/day for a period of 13 weeks. A control group was treated similarly with the vehicle, bidistilled water, only.

The groups comprised 10 animals per sex which were sacrificed after 13 weeks of treatment. Clinical signs, detailed behavioural observations, food consumption and body weights were recorded periodically during the acclimatization and treatment periods. Ophthalmoscopic examinations were performed during the acclimatization and at the end of the treatment period. Functional observational battery, locomotor activity and grip strength were performed during week 13.

At the end of the dosing, blood samples were withdrawn for hematology and plasma chemistry analyses. Urine samples were collected for urinalyses. All animals were sacrificed, 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: All animals survived until scheduled necropsy.

Clinical Signs (Daily and Weekly): No clinical signs of toxicological relevance were noted during daily

observations in males and females at all dose levels.

Detailed Behavioural Observations: No clinical signs were recorded during the weekly detailed behavioral observations (weeks 1-12).

Functional Observational Battery: No clinical signs were recorded during the functional observational battery (week 13).

Grip Strength: No test item-related changes were noted in fore- and hind limb grip strength in male and female rats at any dose level

Locomotor Activity: The mean locomotor activity of males and females was not affected by the treatment with the test item.

Food Consumption: A slight trend to reduced mean daily- and relative food consumption was noted in test item treated animals of both sexes at all dose levels during the treatment period. Although these changes in mean daily- and relative food consumption were not accompanied by changes in body weight development of test item-treated animals, these findings were considered to be related to the treatment with the test item.

Body Weights: The mean body weight development in control and test item-treated animals of both sexes was comparable at any dose level during the treatment period.

Ophthalmoscopic Examinations: Typical background findings (corneal opacity, persistent hyaloid vessel in vitreous body, persistent pupillary membrane) were noted without relationship to dose or treatment.

Clinical Laboratory Investigations: Hematology: After the 13-week treatment period, no test item-related changes of toxicological relevance were noted in hematology parameters in rats of both sexes

at any dose level.

Clinical Biochemistry: After the 13-week treatment period, no test item-related changes of toxicological relevance were noted in clinical biochemistry parameters in rats of both sexes at any dose level.

Urinalysis: After the 13-week treatment period, no test item-related changes of toxicological relevance were noted in the urinalysis in males and females at any dose level.

Organ Weights: There were no differences indicating an effect of the test item. A few statistically significant deviations in average organ weights at the end of the treatment period were considered to be incidental, reflecting the usual individual variability.

Macroscopic / Microscopic Findings: At necropsy, performed at the end of the treatment period, no test item-related macroscopic findings were recorded. The test item, lysophospholipase produced no histological evidence of toxicological properties in the organs and tissues examined.

Conclusion: Oral administration of lysophospholipase to Wistar rats at doses of 100, 300 and 1000 mg/kg/day for at least 13 weeks resulted in no premature death, no clinical signs of adverse nature during daily observations, detailed behavioural observations and during the functional observational battery, no effects on fore- or hind limb grip strength, no effects on locomotor activity, no effects on body weight development, no test item-related changes observed during the ophthalmoscopic examinations, no effects on hematology, clinical biochemistry or urinalysis parameters, no effects on organ weight, no test item-related macroscopic findings of toxicological relevance.

The test item, lysophospholipase produced no histological evidence of toxicological properties in the organs and tissues examined. Insofar as the marginally reduced mean daily absolute and relative food consumption values noted in rats of both sexes were not accompanied by concomitant changes in

mean body weight, and no other findings of toxicological relevance were noted, these differences were considered to be unrelated to the test item.

Therefore, the no-observed effect level (NOEL) and the no-observed-adverse-effect level (NOAEL) were considered to be above 1000 mg/kg/day, the highest dose level used in this study.

C.2.3. 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 lysophospholipase (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 lysophospholipase protein does not shown significant homology to any protein sequence identified or known to be a toxin.

C.3. Information on the Potential Allergenicity of the Enzyme Processing Aid

C.3.1. The source of the Enzyme Processing Aid

The dossier concerns a lysophospholipase from genetically modified *Trichoderma reesei*. The *Trichoderma reesei* host strain is genetically modified to express an *Aspergillus nishimurae* lysophospholipase enzyme.

Name of the enzyme protein: **Lysophospholipase**

Production strain: *Trichoderma reesei* RF7206

Donor:

The lysophospholipase gene, *LPL,* described in this application was isolated from a lambda EMBL3 genomic DNA library of the *Aspergillus nishimurae* (ex-*fumigatus*), using a specific cDNA fragment as a probe. The donor strain was first identified as *Aspergillus fumigatus* and more recently as *Aspergillus nishimurae*. Our *Aspergillus fumigatus* strain is an environmental isolate.

As the name *Aspergillus fumigatus* has been used in our publications, both names *Aspergillus fumigatus* and *Aspergillus nishimurae* are used interchangeably in this dossier for the donor organism.

Aspergillus nishimurae belongs to the section Fumigati of *Aspergillus (Hong et al. 2008)*. The taxonomic lineage of *Aspergillus nishimurae* is shown below (according to http://www.uniprot.org/taxonomy/1220166):

Genus: *Aspergillus*

Species: *Aspergillus nishimurae*

Subspecies (if appropriate): not applicable

Previous or other name(s) (if applicable): *Aspergillus fumigatus*

C.3.2. 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, *LPL* may also cause such occupational allergy in sensitive individuals. However, the possibility of an allergic reaction to the *LPL* 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 *LPL* - 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 likely 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 once reaching the small intestine.
- 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 lysophospholipase enzyme would cross react with known allergens and induce a reaction in an already sensitized individual, sequence homology testing to known allergens was performed.

Alignments of the *LPL* mature amino acid sequence to the sequences in the allergen databases were performed and results obtained were used to estimate the level of potential allergenicity of this enzyme.

Similarity searches were performed to the sequences available in chosen public allergen databases, namely AllergenOnline (FARRP) and Allergen Database for Food Safety (ADFS). Appendix #13 – treated as CCI.

According to the results obtained from the alignments and homology searches it can be concluded that the *LPL* enzyme does not show significant homology to any known allergen. Consequently the risk of *LPL* protein to cause an allergy is regarded as being low.

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 lysophospholipase produced by *Trichoderma reesei* RF7206 under evaluation will cause allergic reactions after ingestion of food containing the residues of these enzymes.

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

- **France**: The enzyme preparation was safety assessed according to the Guidelines for the evaluation of food enzymes (EFSA GL, 2009). This resulted in the authorisation of the enzyme product by the French authorities. The approval letters from the French authorities and the ANSES scientific opinions are included in Appendix #7.
- **USA**: A GRAS determination was notified to the US FDA in 2016 (GRN000653 Appendix #8). In the reply letter from FDA, the agency has no questions regarding AB Enzymes' determination that the lysophospholipase enzyme reparation is GRAS for its intended use.

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 *LPL*, 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*[4].

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[5]**:** 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*.

The parental strain of RF7206 was identified by Centraalbureau voor Schimmelcultures in 2013 as *Trichoderma reesei* (Appendix #13 – treated as CCI).

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 Salomon 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

[4] see: http://amfep.drupalgardens.com/sites/amfep.drupalgardens.com/files/Amfep-List-of-Commercial-Enzymes.pdf
[5] Reference: Mycobank taxonomic database (see: http://www.mycobank.org/Biolomics.aspx?Table=Mycobank&Page=200&ViewMode=Basic).

engineering. The original isolate, QM6a is the initial parent of practically all currently industrially relevant food enzyme production strains, including our strain RF7206.

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[6], if this fungus was to be used in submerged standard industrial fermentation for enzyme production.

[6] reporting procedures in place under the Toxic Substances Control Act (TSCA) for new micro-organisms that are being manufactured for introduction into the commerce.

- 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[7]) 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[8]).

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 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).

[7] http://www.bvl.bund.de/SharedDocs/Downloads/06_Gentechnik/register_datenbanken/organismenliste_2010.pdf?__blob=publicationFile&v=6
[8] http://www.biosafety.be/RA/Class/ClassBEL.html

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 strain RF7206 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 *LPL* fermentation product as produced by the present production strain *Trichoderma* reesei RF7206 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.

Host 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.

The *Trichoderma reesei* host strain is genetically modified with a *LPL* gene deriving from *Aspergillus nishimurae (ex A. fumigatus)*.

Donor

The lysophospholipase gene, *lpl,* described in this application was isolated from a lambda EMBL3 genomic DNA library of the *Aspergillus nishimurae* (ex-*fumigatus*), using a specific cDNA fragment as probe. The donor strain was first identified as *Aspergillus fumigatus* and more recently as *Aspergillus nishimurae*.

Genetic modification

Trichoderma reesei strain RF7206 was constructed for production of *Aspergillus nishimurae* (ex-*fumigatus*) derived *LPL* by introducing the encoding gene into the genome of the *Trichoderma reesei* host.

Standard molecular biology methods were used in the construction of the expression plasmid. The expression cassette fragment used in fungal transformation does not contain any vector derived sequences as it is isolated from the expression plasmid by restriction digestion and purification from an agarose gel.

It consists of a *T. reesei* signal sequence and a carrier polypeptide encoding sequences, the *Aspergillus nishimurae* (ex-*fumigatus*) derived *LPL* coding sequence and *Aspergillus nidulans amdS* gene sequence (as a selection marker).

The DNA fragments that have been transformed to *T. reesei* host strain are well characterized, the sequences of the genes are known, and the fragments are free from any harmful sequences. The transformed DNA does not contain any antibiotic resistance genes.

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 RF7206. Results indicated that several copies of the expression cassettes were integrated in the genome of strain RF7206 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

The food enzyme object of the dossier is typically used in starch processing.

Like any other enzyme, lysophospholipase 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 *LPL* 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[9] (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 lysophospholipase is used:

Foods Uses for Lysophospholipase	
Food Grouping	**Proposed Food Uses**
Sugar products (syrups)	all kind of syrups and sweeteners produced from starch (mainly wheat and maize/corn starches) to be used in beverages, confectionary, etc.)

Application and Raw Material	**Raw Material**	**Maximal recommended use levels (mg TOS/kg RM)**
Starch processing (production of syrups)	Starch (Wheat / corn starch)	1

[9] 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.

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 *LPL* 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	Starch processing (production of syrups)	Starch (Wheat / corn starch)	1	Liquid foods, in which syrups are used, mainly soft drinks	0.15	**0.15**
Solid foods	Starch processing (production of syrups)	Starch (Wheat / corn starch)	1	Solid foods in which syrups are used, e.g. baked products, confectionnary, etc.	0.25	**0.25**

* Assumptions behind ratios of raw material to final food:

Typically:

- starch hydrolysates (sweeteners, syrups) deriving from starch processing are used in a large range of food industries, mainly in soft drinks, dairy, bakery, confectionnary, etc. that fall in the categories of both solid and liquid foods;
- 1 kg of sweetener/syrup is produced per 1 kg starch, meaning that starch hydrolysates (syrups) are 100% starch.

Solid food:

- The most considerable final food applications are dairy and bakery with a maximum added starch content of 5%. Starch is also used in application area of confectionary, where it is used up to a content of 25%. Based upon the highest level of applications (confectionary), the corresponding RM/FF ratio is 0.25 kg starch per kg final food.

Liquid food:

- Syrups and sweeteners are mostly applied in soft drink beverages. Soft drinks typically contain 10-15% w/v HFCS. Therefore, the typical ratio of RM/FF is 0.15 kg starch per L final beverage.

The Total Theoretical Maximum Daily Iintake (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)**
0.25 x 0.0125 = 0.003	0.15 x 0.025 = 0.003	0.006

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 (and beverages) use the specific enzyme lysophospholipase 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 955 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), the Total TMDI of the food enzyme is 0.006mg TOS/kg body weight/day.

Consequently, the MoS is:
MoS = 955 / 0.006 = **159,167**

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 enzyme *LPL* from *Trichoderma reesei* strain RF7206 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 *LPL* from *Trichoderma reesei* strain RF7206 can be used *Quantum Satis* in starch processing.

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 *LPL* enzyme preparation 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 assumed 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

1.1 Customer Support Letter
1.2 Formal Request for Confidential Information (CCI)
1.3 Formal Request for other Confidential Information
1.4 Statutory Declaration
1.5 Checklist 3.1 and 3.3

Section 3.3

1. Product Data Sheets
2. Enzymatic Activity – Confidential
3. Quality Certificates
4. Manufacturing Flow Chart
5. Analytical Data
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7. France Approval
8. USA FDA Approval
9. Pariza and Johnson Decision Tree Analysis
10. AMES

11. Chromosomal aberration
12. 90 Day Oral Toxicity Study
13. CCI Appendix Confidential

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CHECKLIST

(As per section 3.1.11 of the Application Handbook March 1, 2016)

General Requirements (3.1)		
Check Page	**Page No.**	**Mandatory requirements**
☒		**Form of application** ☒Application in English ☒Executive Summary (separated from main application electronically) ☒Relevant sections of Part 3 clearly identified ☒Pages sequentially numbered ☒Electronic copy (searchable) ☒All references provided
☒	9	**Applicant details**
☒	9	**Purpose of the application**
☒	10	**Justification for the application** ☒Regulatory impact information ☒Impact on international trade
☒	11	**Information to support the application** ☒Data requirements
☒	12	**Assessment procedure** ☒General
☒	12	**Confidential commercial information** ☒CCI material separated from other application material ☒Formal request including reasons ☒Non-confidential summary provided
☒	12	**Exclusive Capturable Commercial Benefit** ☒Justification provided
☒	13	**International and other national standards** ☒International standards ☒Other national standards
☒	Appendix 1.4	**Statutory Declaration**
☒	Appendix 1.5	**Checklist/s provided with application** ☒3.1 Checklist ☒All page number references from application included ☒Any other relevant checklists for Chapters 3.2
Processing Aids Requirements 3.2.2		
Check Page	**Page No.**	**Mandatory requirements**
☒	14	A.1 Type of Processing aid

☒	14	A.2 Identification Information
☒	17	A.3 Chemical and Physical Properties
☒	18	A.4 Manufacturing Process
☒	23	A.5 Specification Information
☒	24	A.6 Analytical Method of Detection
☒	24	C.1 Information on Enzyme use on other Countries
☒	25	C.2 Toxicity Information of Enzyme
☒	32	C.3 Allergenicity Information of Enzyme
☒	36	C.4 Overseas Safety Assessment Reports
☒	36	D.1 Information on Source Organism
☒	37	D.2 Pathogenicity and Toxicity of Source Microorganism
☒	40	D.3 Genetic stability of Source Organism
☒	40	E.1 Nature of Genetic Modification of Source Organism
☒	42	F.1 List of Foods likely to Contain the Processing Aid
☒	44	F.2 Anticipated Residue Levels in Foods
☒	47	F.3 Information on likely level of consumption
☒	48	F.4 Percentage of food group to use processing aid
☒	48	F.5 Information on residues in foods in other countries (if available)
☒	48	F.6 Where consumption has changed, information on likely consumption

Statutory Declaration – New Zealand

STATUTORY DECLARATION

Oaths and Declarations Act 1957[2]

I, Karen Lewis, *68440 Eschentzwiller, France,* Chief Financial Officer of AB Enzymes and I, Dr. Gerald Jungschaffer, *Alsbach-Haehnlein, Germany*, Quality Director at AB Enzymes solemnly and sincerely declare that:

1. the information provided in this application fully sets out the matters required; and
2. the information is true to the best of my knowledge and belief; and
3. no information has been withheld which might prejudice this application to the best of my knowledge and belief.

And I make this solemn declaration conscientiously believing the same to be true and by virtue of the *Oaths and Declarations Act 1957*.

Declared at Darmstadt, Germany on 11th January 2018

Signature

Declared before me

[2] http://www.legislation.govt.nz/act/public/1957/0088/latest/DLM314553.html.

AB Enzymes GmbH – Feldbergstrasse 78 , D-64293 Darmstadt

January 29, 2018

Appendix 1.2

Formal Request for Treatment of Confidential Commercial Information (CCI)

AB Enzymes respectfully requests that the selected and marked parts of Appendix 13 are treated as confidential information (CCI).

The information contained in Appendix 13 relates to the construction of the production strain, genetic sequences, introduced DNA, and subsequent technology used. The way an enzyme-producing GMM is constructed is state-of-the-art molecular biochemistry that conditions the productivity of the microorganism and the effectiveness of the enzyme in its uses.

This information is exclusive to AB Enzymes GmbH research and development core technologies and is to be treated as confidential for an unlimited period of time.

Candice Cryne

Regulatory Affairs Manager
AB Enzymes GmbH

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MANILDRA GROUP

Commitment to Excellence

15th May, 2017

AB Enzymes GnbH
Regulatory Affairs
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Letter of Support / Rohalase® F

Manildra herewith would like to support any application seeking FSANZ approval for use in Australia of the new Rohalase® F enzyme preparation from AB Enzymes GmbH.

Based on the first results that have been presented to Manildra, the use of this new enzyme in the starch industry (glucose fiiltration) looks quite promising.

We already foresee this product as being advantageous for our industry and are indeed interested to test the new enzyme at a larger scale in our own facility.

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