A Petition to Amend the Australia New Zealand Food Standards Code with a Triacylglycerol Lipase Enzyme Preparation produced by a genetically modified strain of *Trichoderma reesei* AR-822

AB Enzymes GmbH

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AB Enzymes
an ABF Ingredients company

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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 an enzyme preparation from ***Trichoderma reesei*** *(T. reesei)* host strain genetically modified to produce a ***T. reesei*** **production strain (AR-822) containing a Triacylglycerol Lipase encoding gene** from *Thermomyces lanuginosus*. The enzyme is to be used in the manufacture of:

- bakery products such as, but not limited to bread, steamed bread, bread buns, tortillas, cakes, pancakes, and waffles
- Other cereal-based products

Proposed change to Standard 1.3.3 - Processing Aids

The table **Schedule 18—9(3) Permitted processing aids — various purposes (section 1.3.3—11)**, 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.

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 lipase.

Use of the Enzyme and Benefits

The main activity of the *Trichoderma reesei* AR-822 enzyme preparation is lipase (IUBMB 3.1.1.3). The **function** of lipase is to catalyze the hydrolysis of ester bonds of triacylglycerols (at the position 1 and 3 of the glycerol molecule), resulting in the formation of mono- and diacylglycerols, free fatty acids and, in some cases, also glycerol.

The substrates for the 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.

Triglycerides are found in plants and animals: they are the main constituents of vegetable oils and animal fats. Triglycerides and triacylglycerols are also found for instance in wheat flour: wheat flour contains approximately 2.0–2.5% lipids; wheat lipids can be divided into glycolipids, phospholipids and non-polar lipids (triacylglycerides, mono-glycerides) as shown in Figure 1 below.

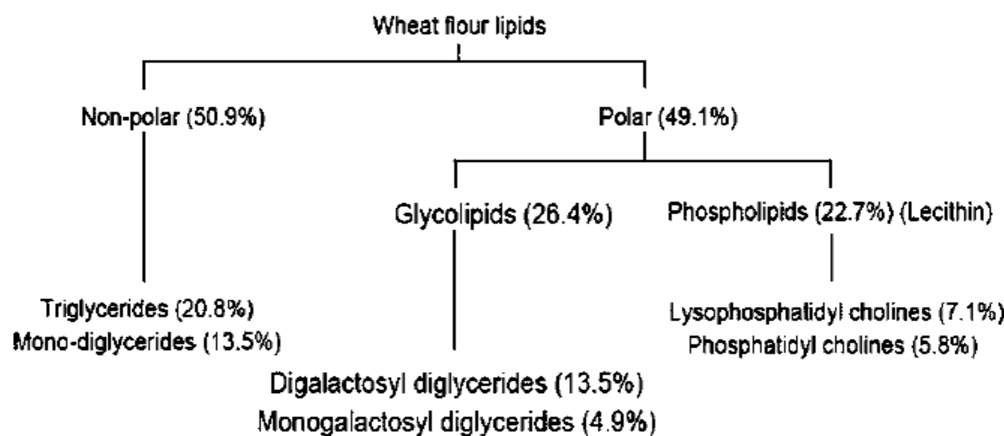

Figure 1: Classification of wheat flour lipids

The reaction products of the hydrolysis of non-polar lipids with the help of lipase are mainly mono- and diacylglycerols and free fatty acids. As the non-polar lipids containing organisms themselves produce lipases, these reaction products are naturally present in these organisms. Consequently, also the reaction products occur naturally in foods.

Lipase activity is widely present in nature and in food ingredients. 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.

The method to analyze 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.

Enzymatic function	catalyze the hydrolysis of ester bonds of triacylglycerols (at the position 1 and 3 of the glycerol molecule)
Substrates	non-polar lipids such as triglycerides or triacylglycerol
Reaction Products	mono- and diacylglycerols and free fatty acids

Like most of the enzymes, lipase performs its technological function during food processing. The lipase from *Trichoderma reesei* AR-822, subject of this dossier, acts as processing aid in the manufacture of bakery products (e.g., bread, biscuits, tortillas, cakes, steamed bread and croissants), and other cereal-based products (e.g., pasta, noodles and snacks). Lipases have been used in baking for the last 30 years and their use in the bakery industry is continuously increasing. This application has been specifically approved for a number of years in Canada, Denmark and France (including the "Pain de tradition Française"), USA, Mexico, Canada, Australia/New Zealand which together with the extensive use for decades demonstrates the technological need of lipases in these food processes.

Baking processes

In baking, lipase performs its technological function during dough or batter handling in order to contribute to an improved and consistent baking process. 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 (Colakoglu and Özkaya 2012)
- Improve dough stability and strength which results in processing tolerance (Rodríguez-García et al. 2014; Colakoglu and Özkaya 2012)
- Improve the dough's structure and behaviour during the baking steps (Rodríguez-García et al. 2014)
- Regulate batter viscosity, beneficial in the production process for e.g., waffles, pancakes and biscuits (Colakoglu and Özkaya 2012; Sîrbu and Pâslaru 2005; Ma et al. 2022)

The process flow of lipase used in the baking process is presented below:

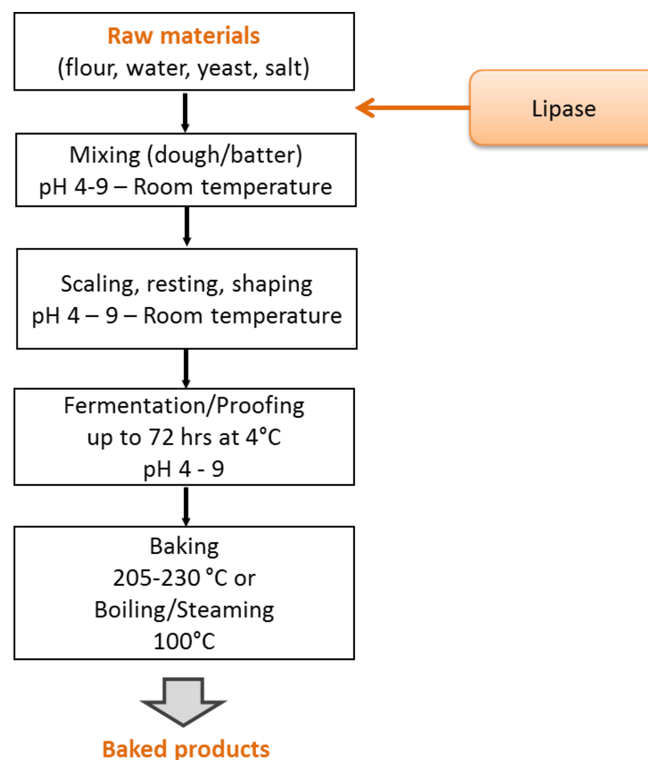

Figure 2: Lipase used in baking products

Other cereal-based processes:

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[1].

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[2].

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).

[1] USA (1970) - US Patent, US 3520702 A "*Method of making dried pasta having a protein network that withstands cooking*")1 available online: http://www.google.com/patents/US3520702

[2] VTT Biotechnology and TNO Nutrition and Food Research Institute (1999) – Second European Symposium on Enzymes in Grain processing - VTT Symposium 207- ESEGP-2 . p. 167 Available online: http://www2.vtt.fi/inf/pdf/symposiums/2000/S207.pdf

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 (Colakoglu and Özkaya 2012)
- Improve dough stability and strength which results in processing tolerance (Gulia et al. 2014)
- Improved texture after boiling/steaming (Ma et al. 2022)

The process flow of lipase used in other cereal-based processes is presented below:

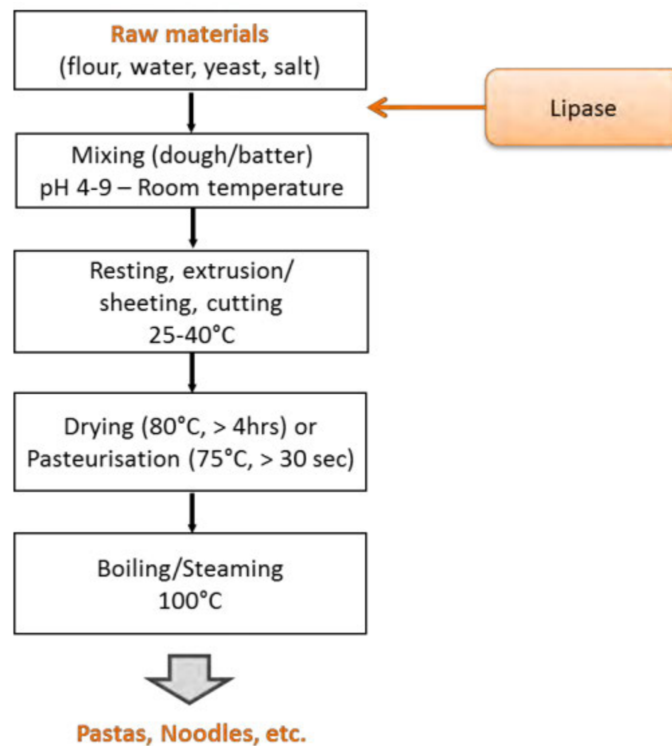

Figure 3: Lipase use in other cereal-based products

Fate of enzyme in food

Like the endogenous lipases present in food raw materials and ingredients, the added lipase does not perform any technological function in the final foods. 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, lack of water

activity, wrong pH, etc. In some cases (e.g., after alcohol distillation), the enzyme may no longer be present in the final food.

In baking, lipases typically perform their technological function during the dough or batter handling. Lipases are denatured by heat during the baking or steaming step.

In cereal-based processes, such as pasta and noodle production, lipases also perform their technological function during dough handling. Afterwards, lipases are denatured by heat during the drying, boiling and/or steaming step.

Consequently, it can be concluded that the lipase does not exert any (unintentional) enzymatic activity in the final foods.

Safety Evaluation

The safety of the lipase produced by the genetically modified *Trichoderma reesei* AR-822 from a toxicological perspective is supported by the historical safety of strain lineage. Toxicological studies were performed on a strain (AR-852) which derives from the same recipient strain as AR-822. Expression constructs are very similar, differing by the expression cassette/enzyme gene of interest. As both production strains are free of any harmful sequences or any potential hazards, the expression cassettes are very similar and are stably integrated into the genome of the strains without any additional mutagenesis cycles thereafter, differences in the genetic modification of AR-822 and AR-852 are not a safety concern. Furthermore, the manufacturing conditions between the two production strains are very similar. The slight changes in pH levels and fermentation medium (food-grade) have been thoroughly assessed. They are considered minor (common industry practice) and do not trigger any additional safety issue.

To add on, the enzyme product from AR-822 production strain complies with JECFA specifications for chemical and microbiological purity of food enzymes (Food and Agriculture Organization of the United Nations 2006) which confirms the safety of the production strain AR-822.

With the use of safe strain lineage, we have substantiated the safety of the AR-822 *Trichoderma reesei* production strain via three toxicological studies on the *Trichoderma reesei* AR-852 production strain to

demonstrate non-toxigenicity of the strain lineage. The toxicological studies conducted include, a reverse mutation assay using bacteria, a Micronucleus Assay in Bone Marrow Cells of the Rat, and a 90-day repeated dose oral toxicity study in Wister rats. All three toxicological studies showed negative findings demonstrating the AR-852 production strain to be non-mutagenic, to not induce structural and/or numerical chromosomal damage, and to not cause toxigenic effects on the Wister rats tested in the 90-day oral toxicity study.

The product is free of production strain and production strain DNA.

AB Enzymes is in the process of registering the *Trichoderma reesei* AR-822 lipase production strain in other countries such as Brazil (ANVISA), EU (EFSA), and USA (US FDA) and planning to register in Canada (Health Canada). Denmark has approved the use of the enzyme.

Conclusion

To conclude, the use of the food enzyme lipase from *Trichoderma reesei* AR-822 in the production of food is safe based on the following aspects presented in this dossier:

- Safety data and information of the production strain
- Allergenicity and toxin analysis assessment on the amino acid sequence of food enzyme
- TDMI value based on Budget Method

Trichoderma reesei has been used in the food industry for many years. Strains from the *Trichoderma reesei* microorganism are generally recognized as safe and are recognized to produce a variety of enzymes. *Trichoderma reesei* is listed as a permitted producer of enzymes in multiple global food enzyme positive lists, including in Australia. The safety of the lipase produced by the genetically modified *Trichoderma reesei* AR-822 from a toxicological perspective is supported by the historical safety of strain lineage which is provided in the dossier. We have demonstrated that the enzyme batches containing lipase from *Trichoderma reesei* AR-822 meet the following criteria:

- Absence of Antibiotic and Toxic Compounds & Analysis of Purity and Identity Specifications of the Enzyme Preparation

- Absence of Production strain
- No Detection of production strain DNA

Based on the safety evaluation, AB Enzymes GmbH respectfully request the inclusion of **Triacylglycerol Lipase** (EC 3.1.1.3) from *Thermomyces lanuginosus* expressed in a genetically modified strain of *Trichoderma reesei* AR-822 in the table of **Schedule 18—9(3) Permitted processing aids — various purposes (section 1.3.3—11)**.

AB Enzymes
an ABF Ingredients company

INTRODUCTION

The dossier herein describes a ***Trichoderma reesei (T. reesei)*** host strain, genetically modified to produce a ***Trichoderma reesei*** **production strain which is non-pathogenic and non-toxicogenic, containing a Triacylglycerol lipase encoding gene** from *Thermomyces lanuginosus*.

Triacylglycerol lipase from *Thermomyces lanuginosus* produced in *Trichoderma reesei* is mainly intended to be used to catalyses the hydrolysis of ester bonds of triacylglycerols, resulting in the formation of mono- and diacylglycerols, free fatty acids and, in some cases, also glycerol.

The following sections describe the genetic modifications implemented in the development of a safe standard host strain, further developed 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 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 lipase enzyme preparation from *Trichoderma reesei* shows no evidence of pathogenic or toxic effects. Estimates of human consumption and an evaluation of dietary exposure are also included.

III. **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
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D-64293 Darmstadt
Germany

Telephone Number

Email Address

Nature of Applicant's Business
Biotechnology

Dossier prepared by

AB Enzymes GmbH

AB Enzymes GmbH

3.1.3. Purpose of the Application

The table **Schedule 18—9(3) Permitted processing aids — various purposes (section 1.3.3—11),** 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).

To explain further, triacylglycerol lipase catalyses the hydrolysis of ester bonds of triacylglycerols, resulting in the formation of mono- and diacylglycerols, free fatty acids and, in some cases, also glycerol.

Triacylglycerol Lipase (EC 3.1.1.3) is a permitted enzyme listed in the table **Schedule 18—9(3) Permitted processing aids — various purposes (section 1.3.3—11)**

3.1.4. Justification for the Application

The need for the proposed change:

Trichoderma reesei expressing a **triacylglycerol lipase** gene from *Thermomyces lanuginosus* is not present as an approved source in the table to **Schedule 18—9(3) Permitted processing aids — various purposes (section 1.3.3—11)**. AB Enzymes GmbH is requesting that this source organism be added. See **Section 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 triacylglycerol lipase enzyme is one of AB Enzymes' latest achievements and has showed great potential in food manufacturing as detailed in the 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 food manufacturers. This is a characteristic that is strongly preferred by manufacturers. There is also a cost benefit associated with the use of *Trichoderma reesei* as superior producer of enzymes resulting in a cost benefit that is passed on to the final user of the enzyme. Increased competition in the market is also a desired characteristic in the context of competition laws. It will increase the choice of 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 food applications.

Due to the effectiveness of this enzyme in the above-mentioned food processes, AB Enzymes has submitted our application in Brazil, EU and USA and plans to register in Canada. Denmark has approved the use of the enzyme.

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 food manufacturers. 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 already have 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 subject 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 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 production strain 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 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 CCI. A summary of this information is given in section E of section 3.2.2. The formal request for treatment of Appendix CCI as confidential commercial information (CCI) is included as Appendix #1.2.

3.1.11. Other Confidential Information

Information related to the approval letters from government authorities is company specific and this information is not publicly available and known only to AB Enzymes GmbH, as such we respectfully ask that this information is kept confidential as presented in Appendix #4. The formal request for treatment of Appendix #4 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 publicly 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 food is not restricted by any Codex Alimentarius Commission (Codex) Standards or any other known regulations.

National Standards:

n/a

3.1.14. Statutory Declaration

The Statutory Declaration is included as Appendices #1.4a and #1.4b.

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 July 2019, 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.

IV. 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

Triacylglycerol lipase is a microbial produced enzyme.

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 as a processing aid in the manufacture of bakery products (e.g., bread, biscuits, tortillas, cakes, steamed bread and croissants), and other cereal-based products (pasta, noodles and snacks). 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	Lipase
Common names	Triacylglycerol acylhydrolase, triacylglycerol ester hydrolase, triacylglycerol lipase
Enzyme Commission No. (IUBMB)	EC 3.1.1.3
CAS number	9001-62-1[3]
Host	*Trichoderma reesei*
Donor	*Thermomyces lanuginosus*

[3] Information on EC 3.2.1.26 - beta-fructofuranosidase - BRENDA Enzyme Database (brenda-enzymes.org)

The classification of the enzyme according to the IUBMB is as follows:

EC 3. is for hydrolyases;

EC 3.1. is for acting on ester bonds;

EC 3.1.1. is for carboxylic-ester hydrolases

EC 3.1.1.3 is for triacylglycerol lipase.

The enzyme is a triacylglycerol lipase enzyme. The food enzyme catalyses the hydrolysis of ester bonds of triacylglycerols, resulting in the formation of mono- and diacylglycerols, free fatty acids and, in some cases, also glycerol. Such enzyme activity is widely present in nature and in particular in food ingredients.

A.2.2. Enzyme Preparation

This dossier includes a triacylglycerol lipase enzyme, produced with the help of *Trichoderma reesei* AR-822 containing a triacylglycerol lipase enzyme gene from *Thermomyces lanuginosus*. The representative current commercial product is VERON® POLARUM.

A.2.3. Final Liquid Enzyme preparation composition

Composition for VERON® POLARUM	
Constituent	**%**
Enzyme concentrate	**5.0 - 10.0**
Sunflower oil	0.4
Wheat flour	Reminder

All substances in the finished enzyme preparation are of food grade quality and conform with the 13th edition of the Food Chemicals Codex (2022) and the *Combined Compendium of Food Additive Specifications* prepared by JECFA.

A.2.4. Enzyme genetic modification

The enzyme is from a *Trichoderma reesei* host strain genetically modified with a triacylglycerol lipase gene deriving from *Thermomyces lanuginosus*. The enzyme is protein engineered.

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® POLARUM

Property	Requirement	
Activity	min.	10,000 ALU/g
Appearance	Light beige to light brown powder	
Particle size distribution	Max 1 % 250 µm	

Lipase catalyses the hydrolysis of ester bonds of non-polar lipids such as triglycerides or triacylglycerol, resulting in the breakdown of non-polar lipids into low levels of mono- and diacylglycerols, free fatty acids and, in some cases also glycerol. Non-polar lipids can be found in founds in plants and animals and they are for instance the main constituents of vegetable oils and animal fats. Consequently, the substrate for lipase occurs naturally in nature and are a natural part of the human diet.

As a result of the catalytic activity of lipase, mono- and diacylglycerols and free fatty acids are formed. These compounds are already present in the human diet. 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.

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 detailed description of the determination of lipase activity is provided in Appendix CCI. The enzyme activity data given above were obtained by this analytical method.

Like most of the enzymes, triacylglycerol 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 several 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,

lack of water activity, suboptimal pH, etc. The enzyme protein triacylglycerol lipase is inactivated by heat in a specific inactivation.

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

Please refer to VERON® POLARUM product data sheet for shelf-life and storage conditions (Appendix #1). Efficacy data of VERON® POLARUM is described in Appendix CCI.

The optimal pH and temperature conditions for the activity of the food enzyme is provided in Appendix CCI.

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

A.3.1. Information on the technological need and mechanism of action of the enzyme in food

Like any other enzyme, the lipase act as a biocatalyst: with the help of the enzyme, a certain substrate is converted into a certain reaction product. The technical effect on the food or food ingredient is caused by the conversion of the substrate to the reaction product caused by the enzymatic reaction involving lipase. Once the conversion occurs, the enzyme can no longer perform a technological function.

The main activity of the *Trichoderma reesei* AR-822 enzyme preparation is lipase (IUBMB 3.1.1.3). The **function** of lipase to catalyze the hydrolysis of ester bonds of triacylglycerols (at the position 1 and 3 of the glycerol molecule), resulting in the formation of mono- and diacylglycerols, free fatty acids and, in some cases, also glycerol.

The substrates for the 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.

Triglycerides are found in plants and animals: they are the main constituents of vegetable oils and animal fats. Triglycerides and triacylglycerols are also found for instance in wheat flour: wheat flour contains approximately 2.0–2.5% lipids; wheat lipids can be divided into glycolipids, phospholipids and non-polar lipids (triacylglycerides, mono-glycerides) as shown in Figure 1 below.

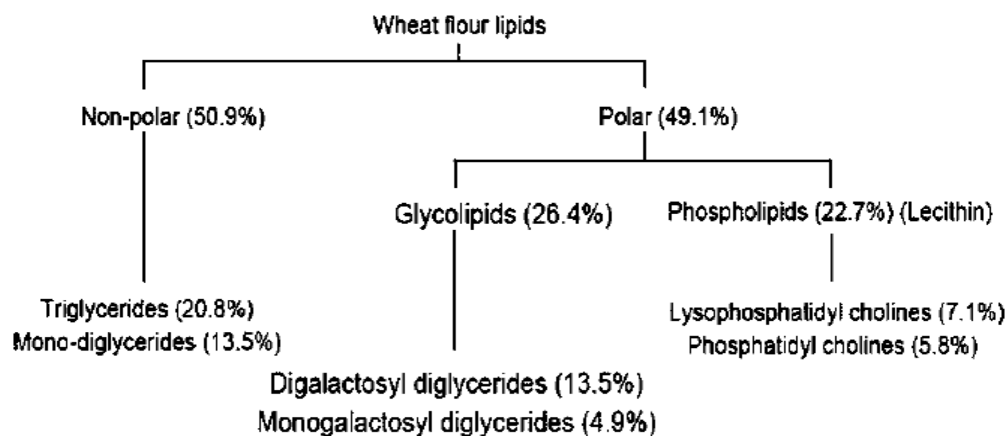

Figure 1: Classification of wheat flour lipids

The reaction products of the hydrolysis of non-polar lipids with the help of lipase are mainly mono- and diacylglycerols and free fatty acids. As the non-polar lipids containing organisms themselves produce lipases, these reaction products are naturally present in these organisms. Consequently, also the reaction products occur naturally in foods.

Lipase activity is widely present in nature and in food ingredients. 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.

The method to analyze 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.

Enzymatic function	Catalyze the hydrolysis of ester bonds of triacylglycerols (at the position 1 and 3 of the glycerol molecule)
Substrates	Non-polar lipids such as triglycerides or triacylglycerol
Reaction Products	Mono- and diacylglycerols and free fatty acids

Like most of the enzymes, lipase performs its technological function during food processing. The lipase from *Trichoderma reesei* AR-822, subject of this dossier, acts as processing aid in the manufacture of bakery products (e.g., bread, biscuits, tortillas, cakes, steamed bread and croissants), and other cereal-based products (e.g., pasta, noodles and snacks). Lipases have been used in baking for the last 30 years and their use in the bakery industry is continuously increasing. This application has been specifically approved for a number of years in Canada, Denmark and France (including the "Pain de tradition Française"), USA, Mexico, Canada, Australia/New Zealand which together with the extensive use for decades demonstrates the technological need of lipases in these food processes.

Baking processes

In baking, lipase performs its technological function during dough or batter handling in order to contribute to an improved and consistent baking process. 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 (Colakoglu and Özkaya 2012)
- Improve dough stability and strength which results in processing tolerance (Rodríguez-García et al. 2014; Colakoglu and Özkaya 2012)
- Improve the dough's structure and behaviour during the baking steps (Rodríguez-García et al. 2014)
- Regulate batter viscosity, beneficial in the production process for e.g., waffles, pancakes and biscuits (Colakoglu and Özkaya 2012; Sîrbu and Pâslaru 2005; Ma et al. 2022)

The process flow of lipase used in the baking process is presented below:

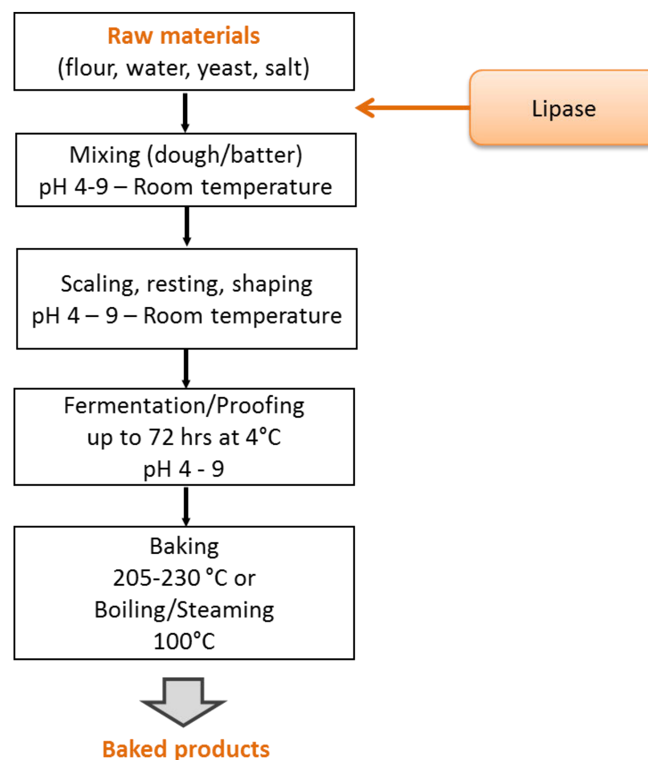

Figure 2: Lipase used in baking products

Other cereal-based processes:

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[4].

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[5].

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).

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[4] USA (1970) - US Patent, US 3520702 A "*Method of making dried pasta having a protein network that withstands cooking*")1 available online: http://www.google.com/patents/US3520702

[5] VTT Biotechnology and TNO Nutrition and Food Research Institute (1999) – Second European Symposium on Enzymes in Grain processing - VTT Symposium 207-ESEGP-2 . p. 167 Available online: http://www2.vtt.fi/inf/pdf/symposiums/2000/S207.pdf

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 (Colakoglu and Özkaya 2012)
- Improve dough stability and strength which results in processing tolerance (Gulia et al. 2014)
- Improved texture after boiling/steaming (Ma et al. 2022)

The process flow of lipase used in other cereal-based processes is presented below:

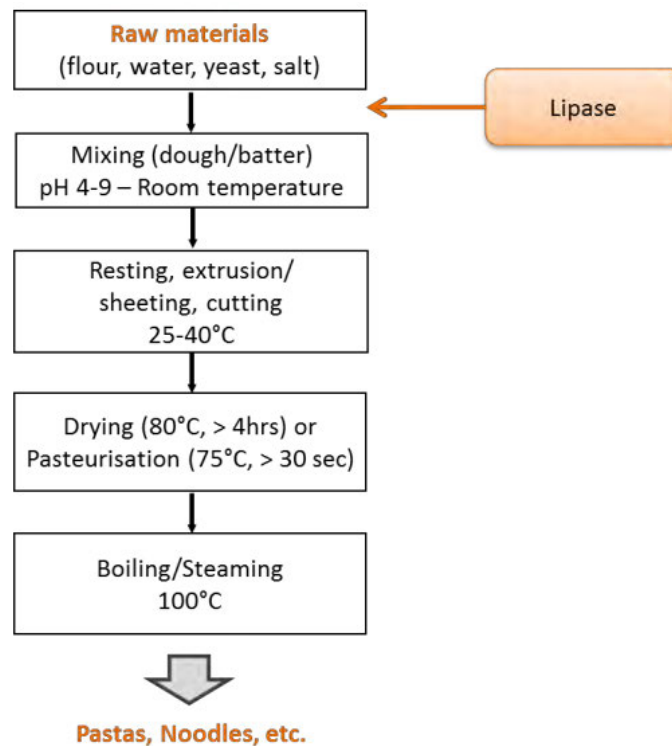

Figure 3: Lipase use in other cereal-based products

Fate of enzyme in food

Like the endogenous lipases present in food raw materials and ingredients, the added lipase does not perform any technological function in the final foods. 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, lack of water

activity, wrong pH, etc. In some cases (e.g., after alcohol distillation), the enzyme may no longer be present in the final food. In order to be able to perform a technological function in the final food, a number of conditions have to be fulfilled at the same time:

- the enzyme protein must be in its 'native' (non-denatured) form, AND
- the substrate must still be present, AND
- the enzyme must be free to move (able to reach the substrate), AND
- conditions like pH, temperature and water content must be favorable

The reasons why the lipase does not exert any (unintentional) enzymatic activity in the final food are:

- the lipase is denatured by heat during the baking or steaming steps of the baking process and during the drying, boiling or steaming steps of processes of other cereal-based products: it is inactivated during regular baking processes, where temperatures inside the dough reach between 95° and 100°C for a period of at least 10-15 minutes.
- the remaining water content (water activity) within baked goods is much too low to support any hydrolytic enzymatic activity in the baking matrix.

In baking, lipases typically perform their technological function during the dough or batter handling. Lipases are denatured by heat during the baking or steaming step.

In cereal-based processes, such as pasta and noodle production, lipases also perform their technological function during dough handling. Afterwards, lipases are denatured by heat during the drying, boiling and/or steaming step.

Consequently, it can be concluded that the lipase does not exert any (unintentional) enzymatic activity in the final foods. Based on the conditions of use and the activity of lipase under such conditions, it can be concluded the presence of (residues of) enzyme lipase in the final food does not lead to an effect in or on the final foods.

A.4. Manufacturing Process

The food enzyme is produced by ROAL Oy through submerged fermentation of *Trichoderma reesei* AR-882 in accordance with current Good Manufacturing Practices for Food (GMP) and the principles of Hazard Analysis of Critical Control Points (HACCP). As it is run in the EU, it is also subject to the Food Hygiene Regulation (852/2004). Quality certificates are provided in Appendix #2.

The enzyme preparation described herein is produced by controlled batch submerged fermentation. The production process involves the fermentation process, recovery (downstream processing) and formulation and packaging. Finally, measures are taken to comply with cGMPs and HACCP. The manufacturing flow-chart is presented in Appendix #3.

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 most cases, the enzyme protein is only partially separated from the other organic material present in the food enzyme.

A.4.1. **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 food enzymes production 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, 13th edition, 2022 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 maximum use levels of antifoam and flocculant are ≤0.15% and ≤1.5% respectively.

The raw materials used for the formulation are of food grade quality. Please refer to Appendix CCI on the composition of the fermentation media specific to produce lipase from *T. reesei* AR-822.

Regarding the use of raw materials that contain known food allergens, the enzyme preparation is formulated with a wheat-based ingredients (as indicated in **Section A.2.3**) which contains gluten.

A.4.3. Materials used in the fermentation process

- Potable water
- A carbon source
- A nitrogen source
- Salts and minerals
- pH adjustment agents
- Foam control agents

A.4.4. Inoculum

A suspension of a pure culture of *T. reesei* AR-822 is aseptically transferred to shake flasks containing fermentation medium. When a sufficient amount of biomass is obtained the shake flasks cultures 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 fermentations are run at a constant temperature and a fixed pH. At the end of the seed fermentation, the inoculum is aseptically transferred to the main fermentor.

A.4.6. Main fermentation

The fermentation in the main fermenter is run as normal submerged fed-batch fermentation. The content of the seed fermenter is aseptically transferred to the main fermenter containing fermentation medium.

In order to control the growth of the production organism and the enzyme production, the feed-rate of this medium is based upon a predetermined profile or on deviation from defined set points.

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 producing microorganisms 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 several types of unit operations described below may vary, depending on the specific enzyme production plant.

A.4.8. **Materials used in recovery process**

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.4.9. **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. Typical amount of filter aids is 2.5 %.

A.4.10. **Primary solid/liquid separation**

The purpose of primary separation is to remove the solids from the enzyme containing fermentation medium. The primary separation is performed at a defined pH and a specific temperature range 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, performed until the desired concentration is obtained. The filtrate containing the enzyme protein is collected for further recovery and formulation.

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. **General Production Controls and Specifications**

To comply with cGMPs and HACCP principles for food production, the following potential hazards in food enzyme production are taken into account and controlled during production as described below:

Identity and purity of the producing microorganism:

Production of the required enzyme protein is based on well-defined Master (MCB) and Working Cell Banks (WCB). The MCB contains the original deposit of the production strain. The WCB is a collection of ampoules containing a pure culture prepared from an isolate of the production strain in MCB. The cell line history, propagation, preservation and the production of a Working Cell Bank is monitored and controlled. A WCB is only accepted for production runs if its quality meets the required standards. This is determined by checking identity, viability, microbial purity and productivity of the WCB. The accepted WCB is used as seed material for the inoculum.

Microbiological hygiene:

Measures to guarantee microbiological hygiene and prevent contamination with microorganisms ubiquitously present in the environment (water, air, raw materials) are as follows:

- Hygienic design of equipment:
- Cleaning and sterilization:
    - Validated standard cleaning and sterilization procedures of the production area and equipment
    - Sterilization of all fermentation media
    - Use of sterile air for aeration of the fermentor
- Hygienic processing:
    - Aseptical transfer of the content of the WCB ampoule, inoculum flask or seed fermentor
    - Maintaining a positive pressure in the fermentor
- Germ filtration

Chemical contaminants:

It is ensured that all raw materials used in production of food enzymes are of food grade quality or have been assessed to be fit for their intended use and comply with agreed specifications.

In addition to these control measures, in-process testing and monitoring are performed to guarantee an optimal and efficient enzyme production process and a high-quality product (cGMPs). The whole process

is controlled with a computer control system which reduces the probability of human errors in critical process steps.

These in-process controls comprise:

Microbial controls:

Absence of significant microbial contamination is analyzed by microscopy or plate counts before inoculation of both the seed and main fermentation and at regular intervals and at critical process steps during fermentation and recovery.

Monitoring of fermentation parameters may include:

- pH
- Temperature
- Aeration conditions

The measured values of these parameters are constantly monitored during the fermentation process. The values indicate whether sufficient biomass or enzyme protein has been developed and the fermentation process evolves according to plan.

Deviations from the pre-defined values lead to adjustment, ensuring an optimal and consistent process.

Enzyme activity and other relevant analyses (like dry matter, refraction index or viscosity):

This is monitored at regular intervals and at critical steps during the whole food enzyme production process.

A.4.14. **Formulation and Packaging**

The enzyme solution before formulation is defined as "food enzyme". Subsequently, the food enzyme is formulated. The resulting product is defined as a 'food enzyme preparation'. For all kinds of food enzyme preparations, the food enzyme is adjusted to the desired activity and is standardized and preserved with food-grade ingredients or additives.

The food enzyme preparation is tested by Quality Control for all related aspects, like expected enzyme activity and the general JECFA Specifications 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.

A.4.15. **Stability of the Enzyme during Storage and Prior to Use**

Food enzymes are formulated into various enzyme preparations to obtain standardized and stable products. The stability thus depends on the type of formulation, not on the food enzyme as such.

The date of minimum durability or use-by-date is indicated on the label of the food enzyme preparation. If necessary, special conditions of storage and/or use will also be mentioned on the label.

A.5. Specification for the Purity and Identity

The food enzyme lipase complies with the internationally accepted JECFA specifications for chemical and microbiological purity of food enzymes (FAO/WHO 2006):

Lead:	No more than 5 mg/kg
Salmonella sp.:	Absent in 25 g of sample
Total coliforms:	Not more than 30 per gram
Escherichia coli:	Absent in 25 g of sample
Antimicrobial activity:	Not detected
Mycotoxins:	No significant levels[6]

Analytical data is provided in Appendix CCI.

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

A.6. Analytical Method for Detection

Please refer to Appendix CCI.

[6] See JECFA specifications, ftp://ftp.fao.org/docrep/fao/009/a0675e/a0675e00.pdf, page 64: Although nonpathogenic and nontoxigenic microorganisms are normally used in the production of enzymes used in food processing, several fungal species traditionally used as sources of enzymes are known to include strains capable of producing low levels of certain mycotoxins under fermentation conditions conducive to mycotoxin synthesis. Enzyme preparations derived from such fungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species.

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

Dossiers have been submitted to the Brazil (ANVISA), EU (EFSA), and USA (US FDA) and there are plans to submit in Canada (Health Canada). Denmark has approved the use of the enzyme (Appendix #4).

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

The safety of *Trichoderma reesei* as an enzyme producer has been reviewed by Frisvad et al. (2018), Nevalainen et al. (1994), Nevalainen et al.; Olempska-Beer et al. (1994; 2006) and Blumenthal (2004). *T. reesei* is regarded as a safe organism for production of industrial enzymes.

Food enzymes, including those derived from recombinant *Trichoderma 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 of dairy products.

At AB Enzymes, *Trichoderma reesei* strains have been used as enzyme producer for many years without any safety problems.

Non-exhaustive list of authorized food enzymes (other than lipase) used *Trichoderma reesei*:

Non-exhaustive list of authorized food enzymes (other than lipase) produced by *Trichoderma reesei*		
Authority	**Food Enzyme**	**Reference**
JECFA	Cellulase Beta-glucanase Glucoamylase	FAS 30-JECFA 39/15 and FAS 22-JECFA 31/31 FAS 22-JECFA 31/25, JECFA monograph gluco amylase
Australia/New Zealand	Cellulase Glucan 1-3 beta-glucosidase Beta-glucanase Hemicellulase complex Gluco-amylase Endo 1,4-beta- xylanase Pectinases	Australia New Zealand Food Standards Code – Schedule 18 – Processing aids (legislation.gov.au)
Canada	Cellulase Glucanase Pentosanase Xylanase Protease Pectinase	5. List of Permitted Food Enzymes (Lists of Permitted Food Additives)
France	Alpha-amylase (GM) Amyloglucosidase (GM) Beta-glucanase (GM) Xylanase Cellulase Lysophospholipase (GM)	Arrêté du 19 octobre 2006
Singapore	Alpha-trehalase Alpha-amylase (GM) Alpha-glucosidase (or maltase) (GM)	Sale of Food Act (sfa.gov.sg)

	Aspergillopepsin I Beta-glucanase Cellulase Xylanase (GM) Glucoamylase Lysophospholipase (GM) Mannanase Pectin lyase (GM) Phospholipase A2 (GM) Polygalacturonase Transglucosidase (GM)	
USA[7]	Pectin lyase Transglucosidase (GM) Glucoamylase Phospholipase A Polygalacturonase Pectin esterase Mannanase Endo-1,4-beta-xylanase Lipase Lysophospholipase Glucose oxidase Serine endopeptidase	GRAS Notice Inventory, GRN 32 GRAS Notice Inventory, GRN 315 GRAS Notice Inventory, GRN 372 GRAS Notice Inventory, GRN 524 GRAS Notice Inventory, GRN 557 GRAS Notice Inventory, GRN 558 GRAS Notice Inventory, GRN 566 GRAS Notice Inventory, GRN 628 GRAS Notice Inventory, GRN 631 GRAS Notice Inventory, GRN 653 GRAS Notice Inventory, GRN 707 GRAS Notice Inventory, GRN 817

[7] GRAS affirmations and GRAS notifications

Non-exhaustive list of authorized lipases from production organisms other than *Trichoderma reesei*:

Non-exhaustive list of authorized lipases from production organisms other than *Trichoderma reesei*		
Authority	**Production Organism**	**Reference**
JECFA	*Aspergillus oryzae*	JECFA Evaluations-LIPASE FROM ASPERGILLUS ORYZAE, VAR.
Australia/NZ	*Aspergillus niger* *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* *Candida rugosa*	Australia New Zealand Food Standards Code – Schedule 18 – Processing aids (legislation.gov.au)
Canada	*Aspergillus niger var.* *Aspergillus oryzae var.* *Rhizopus oryzae var.* *Rhizomucor miehei* *Rhizopus niveus*	5. List of Permitted Food Enzymes (Lists of Permitted Food Additives)
France	Genetically modified *Aspergillus oryzae* containing a gene for a lipase from *Fusarium oxysporum* *Candida rugosa* Genetically modified *Aspergillus niger* containing a gene coding a lipase from *Fusarium culmorum*	Arrêté du 19 octobre 2006
USA[8]	*Penicillium camemberti* *Rhizopus oryzae* produced in *Aspergillus niger*	GRAS Notice Inventory, GRN No. 908

[8] The United States uses a "Generally Considered as Safe" documentation analysis for the acceptance of use for marketing the product

		GRAS Notice Inventory, GRN No. 783

C.2.2. **Toxicological Studies**

This section describes the studies performed to evaluate the safety of the lipase enzyme preparation.

Safe Strain Lineage

Industrial production microorganisms are regularly improved by classical or recombinant DNA methods. If strains from a certain strain lineage have been tested and used for several years, and further improved by e. g. mutagenesis or deleting genes, then one must conclude at a certain point in time that a strain from this strain lineage can be declared safe for use without further testing by extensive programs including animal testing. This strain should be designated as "parental strain" of a "Safe Strain Lineage" and be used as a starting point for further development and improvement for production strains.

Enzyme preparations meet the JECFA definition of Safe Food Enzyme Production Strain[9] or a Presumed Progeny Strain[10] when appropriate toxicological testing (i.e., repeated-dose toxicity and genotoxicity testing) are conducted on enzymes from closely related strains derived from the same parental organism.

As of 2020, JECFA has evaluated over 80 food enzyme preparations from a variety of microorganisms and has never recorded a positive result in any toxicity study, suggesting either toxins were not present or that toxins were present at levels that were below the limit of detection of the bioassays. JECFA concluded that if the introduced genetic modification (either recombinant DNA or chemical mutagenesis) is well characterized, additional toxicological testing would not be required.

[9] A "Safe Food Enzyme Production Strain" is a non-pathogenic, non-toxigenic microbial strain with a demonstrated history of safe use in the production of food enzymes. Evidence supporting this history of safe use includes knowledge of taxonomy, genetic background, toxicological testing, other aspects related to the safety of the strain and commercial food use (Principles Related to Specific Groups of Substances, of Environmental Health Criteria 240 (EHC 240), 2020).
[10] A "Presumed Safe Progeny Strain" is developed from a Safe Food Enzyme Production Strain or from the parent of that Safe Food Enzyme Production Strain. The progeny strain is developed through specific well-characterized modifications to its genome; the modifications must be thoroughly documented, must not encode any harmful substances and must not result in adverse effects. This concept also applies to multiple generations of progeny. Evidence supporting their safety includes knowledge of taxonomy, genetic background and toxicological testing (including read-across of toxicological studies) (Principles Related to Specific Groups of Substances, of Environmental Health Criteria 240 (EHC 240), 2020).

The safety of the lipase produced by the genetically modified *Trichoderma reesei* AR-822 from a toxicological perspective is supported by the historical safety of strain lineage. Toxicological studies. Toxicological studies were performed on a strain (AR-852) which derives from the same recipient strain as AR-822.

The following studies were performed for strain AR-852:

- Reverse Mutation Assay using Bacteria (*Salmonella typhimurium*) with Cellulase produced with *Trichoderma reesei* (Appendix CCI)
- *In vitro* Mammalian Micronucleus Assay in Human Lymphocytes with Cellulase produced with *Trichoderma reesei* (Appendix CCI)
- 90-Day Repeated Dose Oral Toxicity Study in Wister Rats with Cellulase produced with *Trichoderma reesei* (Appendix CCI)

All tests were performed according to the principles of Good Laboratory Practices (GLP) and the current OECD and EU guidelines.

Please refer to the summary of each of the toxicological studies in Appendix #5 and the full studies in Appendix CCI.

As mentioned above both the AR-822 and AR-852 have been developed from the same recipient strain. Expression constructs are very similar, differing by the expression cassette/enzyme gene of interest. As both production strains are free of any harmful sequences or any potential hazards, the expression cassettes are very similar and are stably integrated into the genome of the strains without any additional mutagenesis cycles thereafter, differences in the genetic modification of AR-822 and AR-852 are not a safety concern.

Furthermore, the manufacturing conditions between the two production strains are very similar. The slight changes in pH levels and fermentation medium (food-grade) have been thoroughly assessed. They are considered minor (common industry practice) and do not trigger any additional safety issue.

To add on, enzyme product from AR-822 production strain complies with JECFA specifications for chemical and microbiological purity of food enzymes (Food and Agriculture Organization of the United Nations 2006) which confirms the safety of the production strain AR-822.

Safety of the Production strain (SSL):

For more details on the safety of the *Trichoderma reesei* AR-822 production strain, we refer to the Appendices #5 and #6:

- **Pariza and Johnson Decision Tree**
- **JECFA Safe Progeny Strain statement**
- **Differences between tox tested AR-852 strain and AR-822 production strain**
- **Diagram on Strain Lineage**

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

AB Enzymes conducted a prediction of toxicity of lipase using bioinformatics tools. A homology search was performed from the NCBI Identical Protein Groups (IPG) database using the BLAST-P. The amino acid sequence of the lipase (as shown in Appendix CCI) 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 found in Appendix CCI, it can be concluded that the lipase protein does not show 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 *Thermomyces lanuginosus* expressed in *Trichoderma reesei*.

Name of the enzyme protein: Triacylglycerol lipase

Production strain: *Trichoderma reesei* AR-822

Kingdom: Fungi

Division: *Ascomycota*

Class: *Sordariomycetes*

Order: *Hypocreales*

Family: *Hypocreaceae*

Genus: *Trichoderma*

Species: *Trichoderma reesei*

Strain: *Trichoderma reesei* AR-822

C.4.2. Donor

Name of the Donor: *Thermomyces lanuginosus*

Genus: *Thermomyces*

Species: *Thermomyces lanuginosus*

Subspecies (if appropriate): Not applicable

Commercial name: Not applicable. The organism is not sold as such

The lipase gene described in this application derives from *Thermomyces lanuginosus* (formally known as *Humicola lanuginosa*). *Thermomyces lanuginosus* is considered to be a type of thermophilic fungus (Singh et al. 2003). In Germany, *Thermomyces lanuginosus* is a risk level 1 organism according to BAuA (German Federal Institute for Occupational Safety and Health[11]). Enzymes sourced from *Thermomyces lanuginosus* can be used in industries such as pulp and paper, fats and oils, biofuels, baking among others (Singh et al. 2003; Pedersen and Broadmeadow 2000; Fernandez-Lafuente 2010). Internationally, lipases from *Thermomyces lanuginosus* are recognized as safe, refer to the table below for non-exhaustive list of permitted enzymes from GM strains.

Non-exhaustive list of authorized lipases sourced from *Thermomyces lanuginosus*		
Authority	**Source of Lipase**	**Reference**

[11] BAuA - Advanced search - Federal Institute for Occupational Safety and Health

Brazil	*Thermomyces lanuginosus* expressed in *Aspergillus oryzae* *Thermomyces lanuginosus* and *Fusarium oxysporum* expressed in *Aspergillus oryzae*	RESOLUÇÃO - RDC Nº 728, DE 1° DE JULHO DE 2022 - RESOLUÇÃO - RDC Nº 728, DE 1° DE JULHO DE 2022 - DOU - Imprensa Nacional (in.gov.br)
Australia/ New Zealand	*Aspergillus oryzae,* containing the gene for Lipase, triacylglycerol isolated from *Humicola lanuginosa*[12]	Australia New Zealand Food Standards Code – Schedule 18 – Processing aids (legislation.gov.au)
Mexico	*Aspergillus oryzae* containing the gene for a lipase isolated from *Thermomyces lanuginosus* *Aspergillus oryzae* containing the gene for a lipase isolated from *Thermomyces lanuginosus/Fusarium oxysporum*	ANEXO VI..pdf (www.gob.mx)
Singapore	*Humicola lanuginosa*[12] expressed in *Aspergillus oryzae* *Humicola lanuginosa*[12] and *Fusarium oxysporum* expressed in *Aspergillus oryzae*	Sale of Food Act (sfa.gov.sg)
USA	*Thermomyces lanuginosus* expressed in *Aspergillus oryzae* *Thermomyces lanuginosus* and *Fusarium oxysporum* expressed in *Aspergillus oryzae*	GRAS Notice Inventory, GRN No. 43 GRAS Notice Inventory, GRN No. 103

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

There have been reports of enzymes manufactured for use in food to cause inhalation allergy in workers exposed to the enzyme dust in manufacturing facilities. In the case of lipase, there is a possibility of causing such occupational allergy in sensitive individuals. However, the possibility of an allergic reaction to the lipase residues in food seems remote. To address allergenicity by ingestion of the enzyme, the following may be considered:

- 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*

[12] *Humicola lanuginosa* also known as *Thermomyces lanuginosus*

the food industry". The investigation conducted involved enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and Protein Engineered variants. To add on, the investigation comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. The conclusion from the study was that ingestion of food enzymes in general is not likely to be a concern regarding food allergy.

- In the past, the AMFEP Working Group on Consumer Allergy Risk from Enzyme Residues in Food performed an in-depth analysis of the allergenicity of enzyme food 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.
- Enzymes when used as digestive (Abad et al. 2010) aids are ingested daily, over many years, at much higher amounts when compared to enzymes present in food (up to 1 million times more).

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

There are additional considerations that support the assumptions that the ingestion of enzyme protein is not a concern for food allergy, which are the following:

- 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[13].
- Only a small amount of the food enzyme is used during food processing, which leads to very small amount of enzyme protein present in the final food. A high concentration generally equals a higher risk of sensitization, whereas a low level in final food equals a lower risk (Goodman et al. 2008).
- For cases where the proteins are denatured which is the case for this enzyme due to the food process conditions, the tertiary conformation of the enzyme molecule is destroyed. These types of alterations to the conformation in general, are associated with a decrease in the antigenic reactivity in humans. In the clear majority of investigated human cases, denatured proteins are

[13] The only enzyme protein used in food known to have a weak allergenic potential is egg lysozyme.

much less immunogenic than the corresponding native proteins (Valenta and Kraft 2002; Valenta 2002; Takai et al. 2000; Nakazawa et al. 2005; Kikuchi et al. 2006).

- To add on, residual enzyme still present in the final food will be subjected to digestion in the gastro-intestinal system, that 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 (FAO/WHO 2001; Goodman et al. 2008).

Lastly, 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.

Allergenicity Search

Alignments of the lipase mature amino acid sequence to the sequences in the allergen database were performed and results obtained were used to estimate the level of potential allergenicity of this enzyme. Homology searches were performed to the sequences available in chosen public Allergen Online (FARRP) allergen database version 21 (last updated on February 14, 2021). The allergenicity searches were conducted on February 28, 2023.

The alignment methods used in the searches are:

- Alignment (FASTA) of the entire query amino acid sequence to sequences in allergen online databases.
- Alignment (FASTA) of sliding 80-amino acid windows of the query protein to known protein allergens. Sliding window search means that every possible 80 amino acid segment of the query protein
- 8-mer sequence search

The comparison of query sequence with sequences of known allergens using the sliding 80-mer window was recommended by the FAO/WHO Expert panel already in 2001 and by the Codex Alimentarius Commission in 2003 (Joint FAO/WHO Codex Alimentarius Commission et al. 2009) as a method to evaluate the extent of which a protein is similar in structure to a known allergen.

The identity limit set for the protein having an allergenic cross-reactivity is 35 % when alignment is performed using a full-length query sequence or an 80-mer sliding window. According to EFSA (2010) even the set above 35 % identity is regarded conservative and above 50 % identity cut-off has been suggested.

Results of Allergenicity searches

Type of Search	Outcome
Alignment of the lipase mature amino acid sequence to sequences in allergen online databases	*One hit was identified over the 35 % identity using the full-length search from the AllergenOnline database*, with high E-value (0.99) indicating a non/less significant match – the proteins are not likely to be related in evolution or structure.
Alignment of sliding 80-amino acid window of the query protein to known protein allergens	No matches having greater than 35% identity were found from the AllergenOnline database using the 80-mer sliding window search.
8-mer sequence search	Zero sequences with at least one 8-mer match were detected

To summarize, the bioinformatics approach to estimate potential allergenicity and cross-reactivity based on relatedness to known allergens and taking into account the most recent scientific recommendations on the interpretation of such data leads us to conclude that the **lipase** produced by *Trichoderma reesei* AR-822 is of no concern.

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 to produce lipase is the fungus *Trichoderma reesei.*

Production strain:

Kingdom:	Fungi
Division:	*Ascomycota*
Class:	*Sordariomycetes*
Order:	*Hypocreales*
Family:	*Hypocreaceae*
Genus:	*Trichoderma*
Species:	*Trichoderma reesei*
Strain:	*Trichoderma reesei* AR-822

Taxonomy: *Trichoderma reesei* is a hyper cellulolytic fungus which was found on deteriorating military fabrics such as tents and clothing. This original 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 in synonym with *Trichoderma longibrachiatum* (Bissett 1991). Later however, evidence appeared that the two species were not identical, (Goodfellow et al. 2005; Meyer et al. 1992) and it was decided to go back to the *Trichoderma reesei* name. For a summary of *T. reesei*´s taxonomy, see Druzhinina et al. (2005).

Taxonomic studies have shown that the industrially relevant *Trichoderma reesei* species 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)).

Synonyms[14]**:** *Trichoderma longibrachiatum*

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

D.2. Information on the Pathogenicity and Toxicity of the Source Microorganism

Trichoderma reesei strains are non-pathogenic for healthy humans and animals (Nevalainen et al. 1994; Frisvad et al. 2018). *Trichoderma reesei* is not present on the list of pathogens in the EU (Directive Council Directive 2000/54/EC) and is present in major culture collections worldwide.

***Trichoderma reesei* 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 (NIH, 1998) 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[15], if this fungus was to be used in submerged standard industrial fermentation for enzyme production. To add on in March 2020, the EPA issued a final rule on **Microorganisms; General Exemptions From Reporting Requirements; Revisions to Recipient Organisms Eligible for Tier I and Tier II Exemptions[16]** as part of the 40 Code of Federal Regulations Part 725 where *Trichoderma reesei* is classified as a Tier I organism.
- The Public Health Agency of Canada (PHAC) assigned the species *T. reesei* to 'Risk Group 1' (low individual risk, low community risk) for both humans and terrestrial animals[17]. *T. reesei* is not considered to be an aquatic animal pathogen, nor a regulated plant pest in Canada by the Canadian Food Inspection Agency (CFIA).
- Health Canada's List of Permitted Food Enzymes sets out permitted source organisms (including *T. reesei* A83 (previously named *T. longibrachiatum* A83) and *T. reesei* QM9414 (previously named *T. longibrachiatum* QM9414) for enzymes that may be used as food additives. As per section B.01.045, Part B, of the Food and Drug Regulations, food additives are required to meet

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

[16] https://www.regulations.gov/document?D=EPA-HQ-OPPT-2011-0740-0018

[17] https://www.canada.ca/en/environment-climate-change/services/evaluating-existing-substances/screening-assessment-trichoderma-reesei.html#toc6

specifications set out in these regulations and where no specifications are set out in Part B, the additive must meet specifications set out in the most recent edition of the Food Chemicals Codex (FCC). For food enzymes, the FCC specifications for enzyme preparations would apply.

- FSANZ has approved applications from AB Enzymes in which our *Trichoderma strain* platform has been utilized as the production strain – application A1162 (Lipase from *Trichoderma reesei)*, A1153 (Xylanase from *Trichoderma reeesei*), A1183 (Glucose oxidase from *Trichoderma reesei*), A1238 (Serine endopeptidase from *Trichoderma reesei*).

As a result, *Trichoderma reesei* can be used under the lowest containment level at large scale, GILSP, as defined by OECD (OECD 1992).

The genus *Trichoderma* contains filamentous fungi which are frequently found on decaying wood and in soil. Industrial *T. reesei* strains have a long history of safe use and several of the *Trichoderma* based products have been approved for food and feed applications[18]. *T. reesei* is listed as a "Risk Group 1" organism according to German TRBA classification (Federal Institute for Occupational Safety and Health, www.baua.de) and as "Biosafety Level 1" organism by the American Type Culture Collection (www.atcc.org). *Trichoderma reesei* strains are non-pathogenic for healthy humans and animals. The DNA based identification methods have shown that *T. reesei* is taxonomically different from the other *Trichoderma* species of the section *Longibrachiatum* (Druzhinina et al. 2005).

Some species belonging to *Trichoderma* genus can secrete several types of antibiotics in laboratory cultures. However, strains of *T. reesei* used in industrial applications are proven to be devoid of antibiotic activities (Coenen et al. 1995; Hjortkjaer et al. 1986). The absence of antibiotic activities, according to the specifications recommended by JECFA (FAO/WHO 2006), was also confirmed for AR-822. The analyzed data are presented in Appendix CCI.

Additionally, the original *T. reesei* host and the genetically modified *T. reesei* strain do not carry any acquired antimicrobial resistance genes.

[18] AMFEP. 2009. Association of Manufacturers and Formulators of Enzyme Products List of enzyme products on markets; http://amfep.drupalgardens.com/sites/amfep.drupalgardens.com/files/Amfep-List-of-Commercial-Enzymes.pdf

The production strain is non-toxigenic for the following reasons:

- Results of the toxicological studies provided in the narrative (Appendix #3);
- Safety and history of use of the production organism *Trichoderma reesei;*
- Mycotoxin testing results presented in the composition report (Appendix CCI).

With the use of safe strain lineage, we have substantiated the safety of the AR-822 *Trichoderma reesei* production strain via three toxicological studies on the *Trichoderma reesei* AR-852 production strain to demonstrate non-toxigenicity of the strain lineage. The toxicological studies conducted include, a reverse mutation assay using bacteria, a Micronucleus Assay in Bone Marrow Cells of the Rat, and a 90-day repeated dose oral toxicity study in Wister rats. All three toxicological studies showed negative findings demonstrating the AR-852 production strain to be non-mutagenic, to not induce structural and/or numerical chromosomal damage, and to not cause toxigenic effects on the Wister rats tested in the 90-day oral toxicity study. For more details on the results of the toxicological studies conducted on the production strain, please refer to Appendix CCI.

To add on, as mentioned in this section of the dossier, the *Trichoderma reesei* as a production organism has a long history of use for the production of industrial food enzymes. Food enzymes, including those derived from recombinant *Trichoderma reesei* strains, have been evaluated by JECFA and many countries which regulate the use of food enzymes such as France, Denmark, Australia, and Canada, apart from the USA. Also, AB Enzymes has used *Trichoderma reesei* strains for food enzyme production for many years without any safety problems. Lastly, we have demonstrated the low presence of the mycotoxins produced by the *Trichoderma reesei* microorganism. The composition report provided as an appendix to this GRAS notification demonstrates mycotoxin values below the levels of quantification (LoQs) for the enzyme concentrate batches tested.

Conclusion: Based on the above-mentioned available data, it is concluded that the organism *T. reesei*, has a long history of safe use in industrial-scale enzyme production and can be considered as a safe production organism for enzymes for food as well as feed processing and numerous other industrial applications. As an example, *T. reesei* strains have been cultivated in the production plant of Alko Oy/Roal Oy since 1987. During this time, genetic engineering techniques have been used to improve the industrial

production strains of *Trichoderma reesei* and considerable experience on the safe use of recombinant *Trichoderma reesei* strains at industrial scale has accumulated. Thus, *Trichoderma reesei* and its derivatives can be considered generally safe not only as a production organism of its natural enzymes, but also as a safe host for heterologous gene products. In section 6.1.2 we also provided a short narrative on the safety of the lipase enzyme.

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 blot analysis 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

The *Trichoderma reesei* AR-822 production strain was constructed for lipase production. The construction of the production strain consisted of:

- Expression cassette containing the lipase gene from *Thermomyces lanuginosus* under the control of the *T. reesei* promoter and *Aspergillus nidulans amdS* marker gene
- Introduction of expression cassette into the genome of *Trichoderma reesei* AR-555 recipient strain in one genetic modification step

The expression cassette was cleaved from the pUC19 vector plasmid by restriction enzyme digestion followed by isolation of the expression cassette from agarose gel. A Southern blot hybridization experiment was performed on the genomic DNA of the production strain to confirm that no pUC19 vector DNA was included in the *Trichoderma reesei* AR-822 genome.

Standard DNA techniques were used in the construction of the plasmid and transformation of the expression cassette. The expression cassette was constructed using synthetic gene encoding the lipase enzyme. The transformation of the *T. reesei* recipient strain with the expression cassette containing the lipase gene was performed as described in (Penttilä et al. 1987) with modifications. No vector DNA was

expected to be included in the DNA preparation used for transformation. The transformants were selected according to their ability to grow on acetamide selection plates (*amdS* marker gene).

Detailed information is provided in the Appendix CCI.

E.2. Host/recipient organism

The recipient can be described as followed:

Kingdom: Fungi

Division: *Ascomycota*

Class: *Sordariomycetes*

Order: *Hypocreales*

Family: *Hypocreaceae*

Genus: *Trichoderma*

Species: *Trichoderma reesei*

Strain: *Trichoderma reesei* AR-555

Commercial name: Not applicable. The organism is not sold as such.

E.3. Donor

The *Trichoderma reesei* production strain is genetically modified with lipase gene deriving from *Thermomyces lanuginosus*.

E.4. Genetic modification

T. reesei AR-822 was constructed specifically for lipase production. *T. reesei* AR-822 secretes high amounts of lipase into its culture supernatant, resulting in high lipase activity in the cultivation broth. The produced lipase is the main component of the enzyme mix produced by AR-822. In addition, the strain AR-822 might produce negligible amounts of endogenous *Trichoderma* enzymes. These activities are not relevant from an application/safety point of view, due to the small amount and the fact that such activities have been approved for decades in food processing.

The genetic modification is described in Appendix CCI.

E.5. Stability of the Transformed Genetic Sequence

The stability and potential for transfer of genetic material was assessed as a component of the production microorganism's safety evaluation. Southern blot analyses were performed to the genome of the *T. reesei* production strain AR-822. Further information is described in Appendix 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

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 must 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 this process and determine the amount of enzyme that will provide the desired effect and nothing more. Consequently, from a technological point of view, there are no 'normal or maximal use levels' and **lipase** is used according to the 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.

The dosage of a food enzyme depends on the activity of the enzyme protein present in the final food enzyme preparation (i.e., the formulated food enzyme). However, the activity units do not indicate the amount of food enzyme added.

Microbial food enzymes contain, apart from the enzyme protein in question, also some substances derived from the producing microorganism and the fermentation medium. The presence of all organic materials is expressed as Total Organic Solids (TOS). From a safety point of view, the dosage on basis of TOS is relevant. It must also be noted that the methods of analysis and the expression of the Units are company specific. Consequently, in contrast to when the amount is expressed in TOS activity Units of a certain enzyme cannot be compared when coming from different companies. Because of these reasons, the use levels are expressed in TOS in the table on the next page.

The table below shows the range of recommended use levels for each application where the **lipase** from *Trichoderma reesei* AR-822 may be used:

Food Application	Raw material (RM)	Suggested recommended use levels (mg TOS/kg RM)
Baking and other cereal-based products	Cereals	4

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

The lipase is strictly used as a processing aid and is removed during the production of bakery and/or other cereal-based products by inactivation and dedicated removal steps, as such it is expected that there will be negligible residues. However, to ensure that all requirements are met under the FSANZ guidelines, we have calculated a theoretical maximum potential of the residual enzyme level in foods (choosing the most likely highest type of food consumed) using the Budget Method.

The best method to determine an estimate of human consumption for food enzymes is using the so-called Budget Method (Hansen 1966; Douglass et al. 1997). Through this method, the Theoretical Maximum Daily Intake (TMDI) can be calculated, based on conservative assumptions. These conservative assumptions regard physiological requirements for energy from food and the energy density of food rather than on food consumption survey data.

The original role of the Budget Method was for determining food additive use 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):

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

To determine the TMDI of **lipase** enzyme preparation, the calculation used the maximum use levels. In addition, the calculation accounts for how much food or beverage is obtained per kg raw materials (as shown in the table below), All the TOS is assumed to be in the final product.

Applications		**Raw material (RM)**	**Maximal recommended use level (mg TOS/kg RM)**	**Final food (FF)**	**Ratio RM/FF***	**Maximal level in final food (mg TOS/kg food)**
Solid foods	**Baking and other cereal-based products**	Cereals	4	Baked products	0.71	2.84

*<u>*Assumptions behind ratios of raw material to final food*</u>

<u>Baking:</u>

- *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 crackers, therefore 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 TDMI can be calculated using the maximal values found in food and beverage, multiplied by the average consumption of food and beverage/kg body weight/day. The Total TMDI is the following:

TMDI in food (mg TOS/kg body weight/day)	**TDMI in beverage (mg TOS/kg body weight/day)**	**Total TMDI (mg TOS/kg body weight/day)**
2.84 x 0.0125 = 0.036	0	**0.036**

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 specific lipase from *Trichoderma reesei* AR-822;
- It is assumed that ALL producers apply the HIGHEST use level per application;
- For the calculation of the TMDI's in food, only the above foodstuffs were selected containing the highest theoretical amount of TOS. Therefore, foodstuffs containing lower theoretical amounts were not included;
- 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).

Dietary exposure is calculated on the basis of the total organic solids (TOS) content in the final (commercial) enzyme preparation and is usually expressed in milligrams or micrograms of TOS per kilogram of body weight per day. TOS encompasses the enzyme component and other organic material originating from the production organism and the manufacturing process, while excluding intentionally added formulation ingredients.

The Margin of Exposure (MoE)[19] for human consumption can be calculated through the division of the NOAEL[20] (no-observed adverse effect) value by the TMDI (Total Theoretical Maximal Daily Intake). Total TMDI of the food enzyme is 0.036 mg TOS/kg body weight/day.

As a result, the MoE is:

MoE =1000/0.036 =**28,169**.

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[19] JECFA considers the estimated dietary exposure to an enzyme preparation based on the proposed uses and use levels in food and relates it to the no-observed-adverse-effect level (NOAEL) in its hazard assessment in order to determine a margin of exposure (MoE) section9-1-4-2-enzymes.pdf (who.int)

[20] The NOAEL value is taken from the 90-Day Repeated Dose Oral Toxicity Study in Wister Rats study of the reference production strain AR-852 (see section 6.1.3 for more details).

The value for the Total TMDI is highly exaggerated. In addition, the value for NOAEL was based on the highest dose administered and is therefore considered as a minimum value. Furthermore, the actual Margin of Exposure in practice will be some magnitudes higher. Consequently, there are no safety reasons for laying down maximum levels of use.

Conclusion:

To conclude, the use of the food lipase from *Trichoderma reesei* AR-822 in the production of food is safe. Considering the high safety value determined by the MoE, even when calculating using means of overestimation of intake via the Budget method, there is no need to restrict the use of the enzyme in food. The suggested dosage for food manufacturers is not a restrictive value and could be higher or lower depending on usage within cGMPs.

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.

AB Enzymes
an ABF Ingredients company

V. List of Appendices

Section 3.1

1.1 Customer Support Letter
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Section 3.2

1. Product Data Sheet – VERON® POLARUM
2. Quality Certificates
3. Manufacturing Flow-chart
4. Denmark Approval Letter – other confidential information
5. Summary of Toxicological Studies and Pariza and Johnson Tree
6. Safe Strain Lineage

Appendix CCI – Treated as confidential information

VI. Publication bibliography

Abad, Ana; Fernández-Molina, Jimena Victoria; Bikandi, Joseba; Ramírez, Andoni; Margareto, Javier; Sendino, Javier et al. (2010): What makes Aspergillus fumigatus a successful pathogen? Genes and molecules involved in invasive aspergillosis. In *Revista iberoamericana de micología* 27 (4), pp. 155–182. DOI: 10.1016/j.riam.2010.10.003.

Bindslev-Jensen, Carsten; Skov, Per Stahl; Roggen, Erwin L.; Hvass, Peter; Brinch, Ditte Sidelmann (2006): Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry. In *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 44 (11), pp. 1909–1915. DOI: 10.1016/j.fct.2006.06.012.

Bissett, John (1991): A revision of the genus Trichoderma. II. Infrageneric classification. Canadian Journal of Botany. In *Can. J. Bot.* 69 (11), pp. 2357–2372. DOI: 10.1139/b91-297.

Blumenthal, Cynthia Z. (2004): Production of toxic metabolites in Aspergillus niger, Aspergillus oryzae, and Trichoderma reesei: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. In *Regulatory toxicology and pharmacology : RTP* 39 (2), pp. 214–228. DOI: 10.1016/j.yrtph.2003.09.002.

Coenen, T. M.; Schoenmakers, A. C.; Verhagen, H. (1995): Safety evaluation of beta-glucanase derived from Trichoderma reesei: summary of toxicological data. In *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 33 (10), pp. 859–866.

Colakoglu, Abdullah S.; Özkaya, Hazım (2012): Potential use of exogenous lipases for DATEM replacement to modify the rheological and thermal properties of wheat flour dough. In *Journal of Cereal Science* 55 (3), pp. 397–404. DOI: 10.1016/j.jcs.2012.02.001.

Daurvin, T.; Groot, G.; Maurer, K. H.; Rijke, D. de; Ryssov-Nielsen, H.; Simonsen, M.; Sorensen T.B. (1998): Working Group on Consumer Allergy Risk from Enzyme Residues in Food. AMFEP. Copenhagen.

Douglass, J. S.; Barraj, L. M.; Tennant, D. R.; Long, W. R.; Chaisson, C. F. (1997): Evaluation of the budget method for screening food additive intakes. In *Food additives and contaminants* 14 (8), pp. 791–802. DOI: 10.1080/02652039709374590.

Druzhinina, Irina S.; Kopchinskiy, Alexei G.; Komoń, Monika; Bissett, John; Szakacs, George; Kubicek, Christian P. (2005): An oligonucleotide barcode for species identification in Trichoderma and Hypocrea. In *Fungal genetics and biology : FG & B* 42 (10), pp. 813–828. DOI: 10.1016/j.fgb.2005.06.007.

FAO/WHO (2001): Evaluation of allergenicity of genetically modified foods. Food and Agriculture Organization of the United Nations. Rome, Italy. Available online at http://www.fao.org/3/y0820e/y0820e.pdf.

FAO/WHO (2006): Compendium of food additive specifications. Joint FAO/WHO Expert Committee on Food Additives : 67th Meeting 2006. Rome: FAO (FAO JECFA monographs, 1817-7077, 3). Available online at http://www.fao.org/documents/card/en/c/a6fe72dc-82fb-437c-81cc-bc4d739043a5/.

Fernandez-Lafuente, Roberto (2010): Lipase from Thermomyces lanuginosus: Uses and prospects as an industrial biocatalyst. In *Journal of Molecular Catalysis B: Enzymatic* 62 (3-4), pp. 197–212. DOI: 10.1016/j.molcatb.2009.11.010.

Frisvad, Jens C.; Møller, Lars L. H.; Larsen, Thomas O.; Kumar, Ravi; Arnau, José (2018): Safety of the fungal workhorses of industrial biotechnology. Update on the mycotoxin and secondary metabolite potential of Aspergillus niger, Aspergillus oryzae, and Trichoderma reesei. In *Applied Microbiology and Biotechnology*. DOI: 10.1007/s00253-018-9354-1.

Goodfellow, Michael; Maldonado, Luis A.; Quintana, Erika T. (2005): Reclassification of Nonomuraea flexuosa (Meyer 1989) Zhang et al. 1998 as Thermopolyspora flexuosa gen. nov., comb. nov., nom. rev. In *International journal of systematic and evolutionary microbiology* 55 (Pt 5), pp. 1979–1983. DOI: 10.1099/ijs.0.63559-0.

Goodman, Richard E.; Vieths, Stefan; Sampson, Hugh A.; Hill, David; Ebisawa, Motohiro; Taylor, Steve L.; van Ree, Ronald (2008): Allergenicity assessment of genetically modified crops--what makes sense? In *Nature biotechnology* 26 (1), pp. 73–81. DOI: 10.1038/nbt1343.

Gulia, Neelam; Dhaka, Vandana; Khatkar, B. S. (2014): Instant noodles: processing, quality, and nutritional aspects. In *Critical reviews in food science and nutrition* 54 (10), pp. 1386–1399. DOI: 10.1080/10408398.2011.638227.

Hansen, S. C. (1966): Acceptable daily intake of food additives and ceiling on levels of use. In *Food and cosmetics toxicology* 4 (4), pp. 427–432.

Hjortkjaer, R. K.; Bille-Hansen, V.; Hazelden, K. P.; McConville, M.; McGregor, D. B.; Cuthbert, J. A. et al. (1986): Safety evaluation of Celluclast, an acid cellulase derived from Trichoderma reesei. In *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 24 (1), pp. 55–63.

Joint FAO/WHO Codex Alimentarius Commission; World Health Organization; Food and Agriculture Organization of the United Nations (2009): Foods derived from modern biotechnology. 2nd edition. Rome: World Health Organization : Food and Agriculture Organization of the United Nations (Codex alimentarius, 0259-2916).

Kikuchi, Yuko; Takai, Toshiro; Kuhara, Takatoshi; Ota, Mikiko; Kato, Takeshi; Hatanaka, Hideki et al. (2006): Crucial commitment of proteolytic activity of a purified recombinant major house dust mite allergen Der p1 to sensitization toward IgE and IgG responses. In *Journal of immunology (Baltimore, Md. : 1950)* 177 (3), pp. 1609–1617.

Ma, Mengmei; Mu, Taihua; Sun, Hongnan; Zhou, Liang (2022): Evaluation of texture, retrogradation enthalpy, water mobility, and anti-staling effects of enzymes and hydrocolloids in potato steamed bread. In *Food Chemistry* 368, p. 130686. DOI: 10.1016/j.foodchem.2021.130686.

Meyer, Wieland; Morawetz, Renate; Börner, Thomas; Kubicek, Christian P. (1992): The use of DNA-fingerprint analysis in the classification of some species of the Trichoderma aggregate. In *Current Genetics* 21 (1), pp. 27–30. DOI: 10.1007/BF00318650.

Nakazawa, Takuya; Takai, Toshiro; Hatanaka, Hideki; Mizuuchi, Eri; Nagamune, Teruyuki; Okumura, Ko; Ogawa, Hideoki (2005): Multiple-mutation at a potential ligand-binding region decreased allergenicity of a mite allergen Der f 2 without disrupting global structure. In *FEBS letters* 579 (9), pp. 1988–1994. DOI: 10.1016/j.febslet.2005.01.088.

Nevalainen, H.; Suominen, P.; Taimisto, K. (1994): On the safety of Trichoderma reesei. In *Journal of biotechnology* 37 (3), pp. 193–200.

OECD (1992): Safety Considerations for Biotechnology. ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT, pp. 1–45. Available online at https://www.oecd.org/sti/emerging-tech/2375496.pdf.

Olempska-Beer, Zofia S.; Merker, Robert I.; Ditto, Mary D.; DiNovi, Michael J. (2006): Food-processing enzymes from recombinant microorganisms--a review. In *Regulatory toxicology and pharmacology : RTP* 45 (2), pp. 144–158. DOI: 10.1016/j.yrtph.2006.05.001.

Pedersen, P. B.; Broadmeadow, A. (2000): Toxicological studies on Thermomyces lanuginosus xylanase expressed by Fusarium venenatum, intended for use in food. In *Food additives and contaminants* 17 (9), pp. 739–747. DOI: 10.1080/026520300415273.

Rodríguez-García, Julia; Sahi, Sarabjit S.; Hernando, Isabel (2014): Functionality of lipase and emulsifiers in low-fat cakes with inulin. In *LWT - Food Science and Technology* 58 (1), pp. 173–182. DOI: 10.1016/j.lwt.2014.02.012.

Singh, Suren; Madlala, Andreas M.; Prior, Bernard A. (2003): Thermomyces lanuginosus: properties of strains and their hemicellulases. In *FEMS Microbiol. Rev.* 27 (1), pp. 3–16. DOI: 10.1016/S0168-6445(03)00018-4.

Sîrbu, Alexandrina; Pâslaru, V. (2005): Influence of Lipase Products on Technological Properties of the Bread Flour. In *Scientifical Researches. Agroalimentary Processes and Technologies* 5 (1), pp. 185–192. Available online at https://www.journal-of-agroalimentary.ro/admin/articole/35317L27_Influence_of_Lipase_Products_on_Technological_Properties_of_the_Bread_Flour.pdf.

Takai, T.; Ichikawa, S.; Yokota, T.; Hatanaka, H.; Inagaki, F.; Okumura, Y. (2000): Unlocking the allergenic structure of the major house dust mite allergen der f 2 by elimination of key intramolecular interactions. In *FEBS letters* 484 (2), pp. 102–107.

Valenta, Rudolf (2002): The future of antigen-specific immunotherapy of allergy. In *Nature reviews. Immunology* 2 (6), pp. 446–453. DOI: 10.1038/nri824.

Valenta, Rudolf; Kraft, Dietrich (2002): From allergen structure to new forms of allergen-specific immunotherapy. In *Current opinion in immunology* 14 (6), pp. 718–727.