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A Petition to Amend the Australia New Zealand Food Standards Code with a Fructanase Enzyme Preparation produced by a genetically modified strain of *Trichoderma reesei AR-577*

AB Enzymes GmbH

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I. TABLE OF CONTENTS

II. EXECUTIVE SUMMARY

The present application seeks to amend schedule 18 - Processing Aids of the Australia New Zealand Food Standards Code (the Code) with an enzyme preparation from ***Trichoderma reesei*** *(T. reesei)* host strain genetically modified to produce a ***T. reesei*** **production strain (AR-577) containing a fructanase enzyme** from *Lactobacillus sp*. Based upon the food code, the enzyme is to be used in:

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

Proposed change to Standard 1.3.3 - Processing Aids

The table schedule 18—4(5), **Permitted enzymes (section 1.3.3—6)—Enzymes of microbial origin**, is proposed to be amended to include a genetically modified strain of *Trichoderma reesei* as permitted source for **fructanase** (EC 3.2.1.80).

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 fructanase.

Use of the Enzyme and Benefits

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

The **substrates** for the enzyme are fructo-oligosaccharide (FOS) and related polysaccharides which can be found in cereal and cereal products and therefore occur naturally in nature and are a natural part of the human diet.

The function of the fructanase is to catalyse the hydrolysis of the fructo-oligosaccharide (FOS) and related polysaccharides. FOS and related polysaccharides cause technical difficulties due to its viscosity properties in processing of raw materials containing this component.

Reaction products: as a result of the catalytic activity of fructanase, low levels of FOS and related polysaccharides are present. These compounds are already present in the human diet.

Like most of the enzymes, fructanase performs its technological function during food processing. The fructanase from *Trichoderma reesei* AR-577, object of this dossier, can theoretically be used as processing aid for bakery products.

In general, the technological need of hydrolysis with the help of fructanase can be described as: degradation of a component (the substrate fructo-oligosaccharide (FOS)) which causes technical difficulties in processing of raw materials containing this component.

As described above, fructanase is naturally present in cereal raw materials fermentations by yeast and lactic acid bacteria. The natural enzymatic conversion of fructo-oligosaccharide (FOS) in such materials is of technological benefit in several industrial food manufacturing processes, like baking, etc. However, not all food manufacturing processes can use sourdough fermentation techniques, further the levels of endogenous fructanase are often inadequate and vary from fermentation to fermentation, and the specificity of the enzyme may not be optimal to give desired process advantages. Therefore, industrial fructanse can be used during food processing.

In general, the benefits of FOS hydrolysis with the help of fructanase in baking are:

- Decreasing mixing time of dough
- Increasing level of fermentable and reducing sugars in dough
- Improving yeast fermentation
- Reducing dough viscosity
- Increasing water absorption

Due to better processing conditions, the specific beneficial effects of using fructanase in baking are:

- Facilitating dough handling hence smoother dough

- Helping with gas production by yeast during fermentation
- Reducing added sugar
- Assisting in the formation of flavour and crust colour
- Less product quality variations hereby ensuring standardised quality products

The use of fructanase in food processing will benefit manufacturers through reducing viscosity, improving processability, enhancing yields and shorten processing times, therefore leading to better and/or more consistent product characteristics and more effective production processes. This will eventually result in better production economy and environmental benefits via the use of less raw materials, energy saving and production of less waste, being overall of high value for the food chain.

Safety Evaluation

The safety of the fructanase produced by the genetically modified *Trichoderma reesei* AR-577 from a toxicological perspective is supported by the historical safety of strain lineage. Toxicological studies were performed on a strain (*Trichoderma reesei* AR-852) which derives from the same intermediate strain within the strain lineage of *Trichoderma reesei* AR-577. These results show that there is no need for any toxicological concern with fermentation products as produced by use of *Trichoderma reesei*. Based on the available data, it is concluded that the organism *T. reesei* is non-pathogenic and non-toxigenic and *T. reesei* AR-577 is safe to use as the production organism for fructanase enzyme preparation.

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

The product is free of production strain and recombinant DNA.

AB Enzymes is in the process of registering the *Trichoderma reesei* AR-577 fructanase production strain in other countries such as EU (EFSA).

Conclusion

To conclude, the use of the food enzyme fructanase from *Trichoderma reesei* AR-577 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 homology assessment on 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. Based on safe strain lineage, the results of toxicological studies conducted on a strain from the same lineage - *Trichoderma reesei* AR-852 fructanase production strain are provided in the dossier. We have demonstrated that the enzyme batches containing fructanase from *Trichoderma reesei* AR-577 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 recDNA

Based on the safety evaluation presented herein, AB Enzymes GmbH respectfully request the inclusion of **fructanase** (EC 3.2.1.80) from *Lactobacillus sp.* expressing a genetically modified strain of ***Trichoderma reesei*** AR-577 in the table – 18—4(5), **Permitted enzymes (section 1.3.3—6)—Enzymes of microbial origin**.

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 fructanase enzyme from *Lactobacillus sp***.

Fructanase from *Lactobacillus sp.* expressed in *Trichoderma reesei* is mainly intended to be used in baking processes (e.g., bread, steamed bread, bread buns, tortillas, cakes, pancakes, and waffles).

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

Further sections show the enzymatic activity of the enzyme, along with comparison to other similar enzymes. The safety of the materials used in manufacturing, and the manufacturing process itself is described. The hygienic measurements, composition, and specifications as well as the self-limiting levels of use for fructanase are described. Information on the mode of action, applications, and use levels and enzyme residues in final food products are described. Based on safe strain lineage rationale, the safety studies outlined herein indicate that the fructanase 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

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Telephone Number
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Email Address
[redacted]

Nature of Applicant's Business
Biotechnology

Dossier prepared by
[redacted]

[redacted]

3.1.3. Purpose of the Application

The table schedule 18—4(5), **Permitted enzymes (section 1.3.3—6)—Enzymes of microbial origin**, is proposed to be amended to include a genetically modified strain of *Trichoderma reesei* as permitted source for fructanase (EC 3.2.1.80).

To explain further, fructanase (EC 3.2.1.80) hydrolyses fructans such as inulin, which is a heterogeneous blend of polymers consisting mainly of 2→1 linked fructose units connected to a terminal glucose unit. Similar to inulinase (EC 3.2.1.7), both enzymes hydrolyze terminal, non-reducing 2,1- and 2,6-linked β-D-fructofuranose residues in fructans. However, due to new techniques, there's a taxonomy change in the classification and names of the enzymes. Inulinase (EC 3.2.1.7) is a permitted enzyme listed in the table schedule 18—4(5), **Permitted enzymes (section 1.3.3—6)—Enzymes of microbial origin**.

3.1.4. Justification for the Application

The need for the proposed change:

Trichoderma reesei expressing a fructanase gene from *Lactobacillus sp.* is not present as an approved source in the table to schedule 18—4(5), **Permitted enzymes (section 1.3.3—6)—Enzymes of microbial origin** (included in the table as Inulinase). 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 fructanase 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 bakery product manufacturers. This is a characteristic that is strongly preferred by manufacturers. There is also a cost benefit associated with the use of *Trichoderma reesei* as superior producer of enzymes resulting in a cost benefit that is passed on to the final user of the enzyme. Increased competition on the market is also a desired characteristic in the context of competition laws. It will increase the choice to local manufacturers and help in reducing production costs as compared to the currently known and marketed products of the same enzyme class used for the same type of bakery applications.

Due to the effectiveness of this enzyme in the above-mentioned food processes, AB Enzymes is in the process of submitting application in the EU and plans to submit in Denmark, USA, Canada and Mexico.

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—4(5) is not intended to place any costs or regulatory restrictions on industry or consumers. Inclusion of the enzyme will provide food manufacturers with an alternative. For government, the burden is limited to necessary activities for a variation of Standard 1.3.3.

3.1.7. Impact on International Trade:

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

3.1.8. Information to Support the Application

Public Health and Safety Issues related to the Proposed Change:

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

The safety of fructanase, food enzyme object of the present dossier, produced by the genetically modified *Trichoderma reesei* AR-577 from a toxicological perspective is supported by the historical safety of strain lineage. Toxicological studies were performed on a strain (*Trichoderma reesei* AR-852) which derives from the same intermediate strain within the strain lineage of *Trichoderma reesei* AR-577. Toxicological tests that have been performed included a 90-day repeated dose study. These results show that there is no need for any toxicological concern with fermentation products as produced by use of *Trichoderma reesei*. Based on the available data, it is concluded that the organism *T. reesei* is non-pathogenic and non-toxigenic and *T. reesei* AR-577 is safe to use as the production organism for fructanase enzyme preparation.

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

The product is free of production strain and recombinant DNA.

Consumer choice related to the Proposed Change:

Consumer choice is not expected to be changed directly as the enzyme is used as a processing aid and is not purchased by consumers. Fructanase 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 fructanase 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 (refer to **Section 3.1.3.**), 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

n/a

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 bakery products 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 Appendix #1.3a and 1.3b.

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 #1.4.

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

Fructanase 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 fructanase in bakery products. 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	β-D-fructan fructohydrolase
Common names	fructanase, fructan β-fructosidase, exo-β-D-fructosidase, exo-β-fructosidase, fructan exohydrolase, polysaccharide β-fructofuranosidase
Enzyme Commission No. (IUBMB)	EC 3.2.1.80
CAS number	37288-56-5[1]
Host	*Trichoderma reesei* AR-577
Production strain of Host	AR-557
Donor	*Lactobacillus sp.*

[1] EC 3.2.1.80 (qmul.ac.uk)

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

EC 3. is for hydrolases;

EC 3.2. is for glycosylases;

EC 3.2.1. is for glycosidases, i.e., enzymes hydrolysing *O*- and *S*-glycosyl compounds;

EC 3.2.1.80 is for fructan beta-fructosidase.

A.2.2. **Enzyme Preparation**

This dossier includes a fructanase enzyme, produced with the help of *Trichoderma* reesei AR-577 containing a fructanase enzyme gene from *Lactobacillus sp*. The representative current commercial product is VERON® FR.

A.2.3. **Enzyme preparation composition**

Composition for VERON® FR	
Constituent	**%**
Fructanase Enzyme concentrate	**2-4**
Sunflower oil	0.2
Wheat Flour	Remainder

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 an fructanase gene deriving from *Lactobacillus sp.* The enzyme is considered 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® FR

Property	Requirement	
Activity	min.	600FRU/g
Particle size distribution	Max 1% > 250 µm	
Appearance	Light Beige powder	

The approximate molecular weight of the fructanase enzyme is 120 kDa.

The substrates and the reaction products are themselves present in food ingredients. No reaction products which could not be considered normal constituents of the diet are formed during the production or storage of the enzyme treated food. Consequently, no adverse effect on nutrients is expected.

Like most of the enzymes, fructanase performs its technological function during food processing and does not perform any technological function in the final food. The reasons why the enzyme does not exert any (unintentional) enzymatic activity in the final food can be due to a combination of various factors, depending on the application and the process conditions used by the individual food producer. These factors include depletion of the substrate, denaturation of the enzyme during processing (which is clearly the case during baking process), lack of water activity, wrong pH, etc. The enzyme protein fructanase is inactivated by heat in a specific inactivation step or in a sterilisation/pasteurization process or further drying steps.

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

Please refer to VERON® FR product data sheet for shelf-life and storage conditions (Appendix #1).

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 fructanase acts as a biocatalyst: with the help of the enzyme, a certain substrate is converted into a certain reaction product. It is not the food enzyme itself, but the result of this conversion that determines the effect in the food or food ingredient. After the conversion has taken place, the enzyme no longer performs a technological function. The main activity of the *T. reesei* AR-577 enzyme preparation is fructanase (IUBMB 3.2.1.80).

The **substrates** for the enzyme are fructo-oligosaccharide (FOS) and related polysaccharides which can be found in cereal and cereal products and therefore occur naturally in nature and are a natural part of the human diet.

The function of the fructanase is to catalyse the hydrolysis of the fructo-oligosaccharide (FOS) and related polysaccharides. FOS and related polysaccharides cause technical difficulties due to its viscosity properties in processing of raw materials containing this component.

Reaction products: as a result of the catalytic activity of fructanase, low levels of FOS and related polysaccharides are present. These compounds are already present in the human diet.

Like most of the enzymes, fructanase performs its technological function during food processing. The fructanase from *Trichoderma reesei* AR-577, object of this dossier, can theoretically be used as processing aid for bakery products.

Fructo-oligosaccharide (FOS) and related polysaccharides are a natural part of the human diet, as they are found in cereals. Consequently, the substrate for fructanase occurs naturally in cereal-based foods. Like the substrate, the enzyme also occurs by nature in yeast and many lactic acid bacteria found in sourdough applications but in inconsistent or too low amounts for the function needed in the processes described in this dossier. 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 use of fructanase in food processing will benefit manufacturers through reducing viscosity, improving processability, enhancing yields and shorten processing times, therefore leading to better and/or more consistent product characteristics and more effective production processes. This will eventually result in better production economy and environmental benefits via the use of less raw materials, energy saving and production of less waste, being overall of high value for the food chain.

Like most enzymes, the fructanase performs its technological function during food processing. The fructanase from *T. reesei* AR-577 object of this dossier is specifically intended to be used in **baking** (e.g., bread, bread buns, tortillas, crackers etc.). In these processes, the fructanase is used as a processing aid in food manufacturing and is not added directly to final foodstuffs.

Below, the benefits of the use of industrial fructanase in baking are described.

Baking Process

Fructanase can be used in the manufacturing of bakery products such as, but not limited to bread, steamed bread, bread buns, tortillas, cakes, pancakes, and waffles.

In general, the technological need of hydrolysis with the help of fructanase can be described as: degradation of a component (the substrate fructo-oligosaccharide (FOS)) which causes technical difficulties in processing of raw materials containing this component.

As described above, fructanase is naturally present in cereal raw materials fermentations by yeast and lactic acid bacteria. The natural enzymatic conversion of fructo-oligosaccharide (FOS) in such materials is of technological benefit in several industrial food manufacturing processes, like baking, etc. However, not all food manufacturing processes can use sourdough fermentation techniques, further the levels of endogenous fructanase are often inadequate and vary from fermentation to fermentation, and the specificity of the enzyme may not be optimal to give desired process advantages. Therefore, industrial fructanse can be used during food processing.

As explained above, fructanase is acting on hydrolysis of fructo-oligosaccharide (FOS) and related polysaccharides and is very often used together with other enzymes (enzyme systems). In particular,

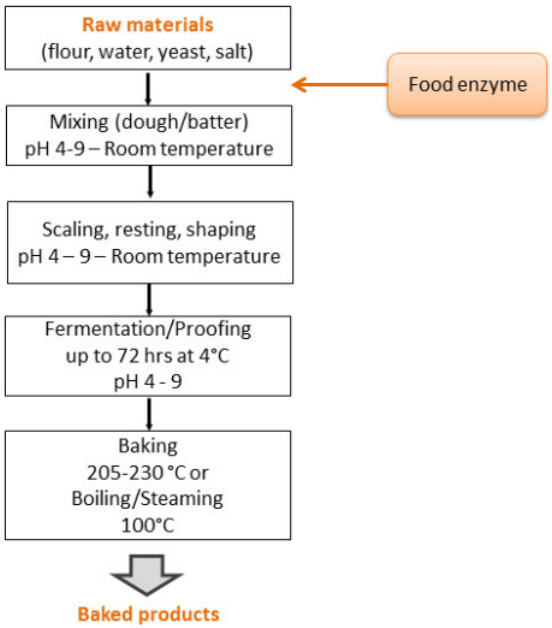
fructanase is used in combination with alpha-amylases, maltogenic amylases, xylanases, lipases and proteases.

In this process, the use of fructanase assists the food processing (such as reducing the viscosity and improving processability, enhancing yields, shorten processing times etc.) therefore leading to better and/or more consistent product characteristics and helping to achieve more effective production processes, resulting in better production economy and environmental benefits such as the use of less raw materials, energy saving and production of less waste, being overall of high value for the food chain.

In general, the benefits of FOS hydrolysis with the help of fructanase in baking are:

- Decreasing mixing time of dough
- Increasing level of fermentable and reducing sugars in dough
- Improving yeast fermentation
- Reducing dough viscosity
- Increasing water absorption

Please refer to the process flow below demonstrating how fructanase is used in the manufacture of baking products:



Due to the better processing, the specific beneficial effects of using **fructanase** in baking are:

- Facilitating dough handling hence smoother dough
- Helping with gas production by yeast during fermentation
- Reducing added sugar
- Assisting in the formation of flavour and crust colour
- Less product quality variations hereby ensuring standardised quality products

Fate of the Enzyme in Baking

As explained, it is not the food enzyme itself, but the result of the enzymatic conversion that determines the effect in the food or food ingredient (including raw materials). This effect remains, irrespective of whether the food enzyme is still present or removed from the final food.

Fructanase performs its technological function during food processing. In some cases, the enzyme may no longer be present in the final food. In other cases, where the enzyme protein is still present in the final food, it does not perform any technological function in the final food, just like the endogenous fructanase in baking raw materials and ingredients.

For fructanase 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 favourable

In baking, the fructanase, performs its technological function during the first steps of the baking process. The fructanase is denatured by heat during baking (when higher temperatures above 80°C is reached) and has no further technological effect after baking.

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

A.4. Manufacturing Process

The food enzyme is produced by ROAL Oy by submerged fermentation of *Trichoderma reesei* AR-577 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 #4.

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

A.4.1. **Fermentation**

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

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

- Inoculum
- Seed fermentation
- Main fermentation

A.4.2. **Raw materials**

The raw materials used in the fermentation and recovery processes are standard ingredients that meet predefined quality standards controlled by Quality Assurance for ROAL Oy. The safety is further confirmed by toxicology studies (See **Section C**). The raw materials conform to either specifications set out in the Food Chemical Codex, 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 flocculants are ≤0.15% and ≤1.5% respectively.

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

A.4.3. Materials used in the fermentation process (inoculum, seed and main fermentation)

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

For details regarding fermentation ingredients, please refer to Appendix CCI.

A.4.4. Inoculum

A suspension of a pure culture of *T. reesei* AR-577 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

After sufficient growth, the biomass is aseptically transferred to a seed fermentor, where further growth takes place under agitation and aeration at a constant temperature and a fixed pH.

A.4.6. Main fermentation

Biosynthesis of the fructanase enzyme product by the production strain *T. reesei* AR-577 occurs during the main fermentation.

The contents of the seed fermentor are aseptically transferred into the main fermentor, where enzyme production will take place. The main submerged fermentation is run under specified pH, temperature and aeration conditions, until sufficient enzyme production has taken place.

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

A.4.7. **Recovery**

The purpose of the recovery process is:

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

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

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

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

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

A.4.8. **Materials**

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

- Flocculants

- Filter aids
- pH adjustment agents

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

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 the 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, which is performed until the desired concentration has been 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:

The assurance that the production microorganism efficiently produces the desired enzyme protein is of utmost importance to the food enzyme producer. Therefore, it is essential that the identity and purity of the microorganism is controlled.

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:

For optimal enzyme production, it is important that hygienic conditions are maintained throughout the entire fermentation process. Microbial contamination would immediately result in decreased growth of the production organism, and consequently, in a low yield of the desired enzyme protein, resulting in a rejected product.

Measures utilized by ROAL OY 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:
    - all equipment is designed, constructed and used to prevent contamination by foreign micro-organisms
- 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 fermentors
- 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

In parallel, hygienic conditions in production are furthermore ensured by:

- Training of staff:
  - all the procedures are executed by trained staff according to documented procedures complying with the requirements of the quality system.
- Procedures for the control of personal hygiene
- Pest control
- Inspection and release by independent quality organization according to version-controlled specifications
- Procedures for cleaning of equipment including procedures for check of cleaning efficiency (inspections, flush water samples etc.) and master cleaning schedules for the areas where production takes place
- Procedures for identification and implementation of applicable legal requirements
- Control of labelling
- Requirements to storage and transportation

Chemical contaminants:

It is also important that the raw materials used during fermentation are of suitable quality and do not contain contaminants which might affect the product safety of the food enzyme and/or the optimal growth of the production organism and thus enzyme yield.

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 is 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**

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.

Fructanase enzyme preparation from *T. reesei* AR-577 is sold mainly as a powdered product.

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

It is proposed that the food enzyme fructanase should comply 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[2]

Analytical data and methods used are provided in **Table 3** and Appendix CCI.

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

A.6. Analytical Method for Detection

Please refer to Appendix CCI.

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

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

[2] 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.

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 is being submitted to the EU (EFSA) and planned to be submitted in Denmark (DVFA), Canada (Health Canada), Mexico (COFEPRIS) and USA (FDA).

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 genetic modification, i.e., the transformation of the recipient strain *Trichoderma reesei* with the plasmid results in recombinant strain AR-577. As mentioned before, the recipient strain belongs to a non-pathogenic species. The strain line has been used since 2010 for safe food enzyme production.

Fructanase (EC 3.2.1.80) hydrolyses fructans such as inulin, which is a heterogeneous blend of polymers consisting mainly of 2→1 linked fructose units connected to a terminal glucose unit. Similar to inulinase (EC 3.2.1.7), both enzymes hydrolyze terminal, non-reducing 2,1- and 2,6-linked β-D-fructofuranose residues in fructans. However, due to new techniques, there's a taxonomy change in the classification and names of the enzymes.

Commercial fructanase and Inulinase enzyme preparations from various microorganisms (including genetically modified ones) are widely accepted and *Trichoderma reesei* – whether or not genetically modified - is widely accepted as a safe production organism for a broad range of enzymes that have been used e.g., as processing aids in food industry for several decades.

Non-exhaustive list of authorized fructanase and inulinase from similar production organisms		
Authority	**Production Organism**	**Reference**
Australia/NZ	Inulinase (EC 3.2.1.7) from *Aspergillus niger*	Schedule 18 Processing Aids
Brazil	Inulinase from *Aspergillus niger* *Kluyveromyces fragilis*	RESOLUÇÃO DA DIRETORIA COLEGIADA - RDC Nº 53, DE 7 DE OUTUBRO DE 2014

	Sporotrichum dimorphosporum	
Canada	Inulinase from *Aspergillus niger* var. Tieghem	5. List of Permitted Food Enzymes
France	Inulinase of non-genetically modified strain of *Aspergillus niger*	Arrêté du 19 octobre 2006
Indonesia	Inulase; indo-inulinase; endo-inulinase; exo-inulinase; 2,1-β-D-fructan fructanohydrolase; 1-β-D-fructan fructanohydrolase from *Aspergillus niger* *Disporotrichum dimorphorsporum* *Kluyvercmyces fragilis*	BPOM No.28 Year 2019 Processing Aids in Processed Foods
India	Inulinase of *Aspergillus niger*	Food Safety and Standards (Food Products Standards and Food Additives) Ninth Amendment Regulations, 2020
Singapore	Inulinase of *Aspergillus niger*	Sale of Food Act Eighth Schedule

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

C.2.2. **Toxicological Studies**

This section describes the studies performed to evaluate the safety of the fructanase 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[3] or a Presumed Progeny Strain[4] 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.

[3] 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).

[4] 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 use of safe strain lineage concept is only a waiver for toxicological studies, however it does not negate the enzyme product from other safety requirements, such as allergenicity, cytotoxicity, toxin analysis and other safety parameters.

The fructanase is produced by *Trichoderma reesei* AR-577 production strain. The transformation of the recipient strain AR-329 *Trichoderma reesei* with the expression cassette results in recombinant strain AR-577 (see **Table 1** below).

The production organism *Trichoderma reesei* has been genetically engineered by deleting genes from the genome and by transformation of the strain with the expression plasmid to promote fructanase production. All genetic modifications are well characterized and as such the safe strain lineage concept was employed as the *Trichoderma* reesei intermediate strain, AR-288, was similarly used for the AR-852 strain which has been assessed in several toxicological studies as presented below in **Table 1 and Table 2**. AR-288 is the last common intermediate strain after which the lineage separates. Both AR-577 and AR-852 derive from AR-288. The recipient strain for AR-577, AR-329 contains one more deletion than the recipient strain for AR-852, AR-555. The additional deletion is minor and done to further reduce the already low side activities. The safe strain lineage flow chart is present in **Figure 1** illustrating the relationship between the different strains in the lineage. The differences between the two strains (AR-577 and AR-852) are the inserted expression cassettes containing the enzyme genes of interest and the additional deletion applicable to AR-577.

Table 1: Comparison of the AR-577 and Toxicological tested strain AR-852 Expression Cassettes

Production Strain	Promoter[5]	Signal Sequence	Enzyme	Terminator[6]	Selection marker
AR-577 *T. reesei*	*T. reesei* promoter	Native fructanase signal sequence	Fructanase	*T. reesei* terminator	*A. nidulans* amdS

[5] Expression cassettes for AR-577 and AR-852 use different promoters native to *T. reesei*

[6] Expression cassettes for AR-577 and AR-852 use different terminators native to *T. reesei*

AR-852 *T. reesei*	*T. reesei* promoter	Native cellulase signal sequence	Cellulase	*T. reesei* terminator	*A. nidulans* amdS

Table 2: Toxicological Test Summaries

Production Strain	Enzyme	Toxicology Test	Result
AR-852 *T. reesei*	Cellulase	90-Day Repeated Dose Oral Toxicity Study in Wistar Rats	No adverse effects
		Reverse Mutation Assay using Bacteria (*Salmonella typhimurium and E. coli*)	Non-mutagenic
		In vitro Mammalian Micronucleus Assay in Human Lymphocytes	Non-clastogenic

Toxicological Studies of *Trichoderma reesei* AR-852

The safety of the fructanase produced by the genetically modified *Trichoderma reesei* AR-577 from a toxicological perspective is supported by the historical safety of strain lineage. Toxicological studies were performed on a strain (*Trichoderma reesei* AR-852) which derives from the same intermediate strain within the strain lineage of *Trichoderma reesei* AR-577.

The following studies were performed for strain *Trichoderma reesei* 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 Wistar 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.

As mentioned above both the AR-577 and AR-852 have been developed from the same intermediate strain. Expression constructs are very similar, only 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 growth/mutagenesis cycles thereafter, differences in the genetic modification of AR-577 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-577 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-577.

Safety of the Production strain (SSL):

For more details on the safety of the *Trichoderma reesei* AR-577 production strain, we refer to Appendix #3 for the following explanation:

- **Pariza and Johnson Decision Tree**
- **JECFA Safe Progeny Strain statement**
- **Differences between tox tested strain and AR-577 production strain**
- **Diagram on Strain Lineage**

Please refer below for the summary of each of the toxicological study from safe strain lineage AR-852.

C.2.2.1. Reverse Mutation Assay using Bacteria Test (*Salmonella typhimurium*) with Cellulase produced with *Trichoderma reesei*

The test was conducted in line with the OECD Guidelines for Testing of Chemicals, Section 4, No. 471, "Bacterial Reverse Mutation Test". In order to investigate the potential of Cellulase produced with *T. reesei* for its ability to induce gene mutations the plate incorporation test (experiment I) and the pre-incubation

test (experiment II) were performed with the *Salmonella typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and TA 102.

In two independent experiments several concentrations of the test item were used. Each assay was conducted **with** and **without** metabolic activation) in experiment I and II.

No precipitation of the test item was observed in any tester strain used in experiment I and II (**with** and **without** metabolic activation).

No toxic effects of the test item were noted in any of the five tester strains used up to the highest dose group evaluated (**with** and **without** metabolic activation) in experiment I and II.

No biologically relevant increases in revertant colony numbers of any of the five tester strains were observed following treatment with Cellulase produced with *T. reesei* at any concentration level, neither in the nor absence of metabolic activation in experiment I and II.

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, Cellulase produced with *T. reesei* did not cause gene mutations by base pair changes or frameshifts in the genome of the tester strains used.

Therefore, Cellulase produced with *T. reesei* is considered to be non-mutagenic in this bacterial reverse mutation assay.

C.2.2.2. *In vitro* Mammalian Micronucleus Assay in Human Lymphocytes with Cellulase produced with *T. reesei*

The test was conducted in line with OECD Guidelines for Testing of Chemicals, Section 4, No. 487, "In Vitro Mammalian Cell Micronucleus Test". In order to investigate a possible potential of Cellulase produced with *T. reesei* to induce micronuclei in human lymphocytes an *in vitro* micronucleus assay was carried out. The following study design was performed:

	Without S9		**With S9**
	Exp. 1	**Exp. II**	**Exp. I**
Exposure period	4 h	44 h	4 h
Cytochalasin B exposure	40 h	43 h	40 h
Preparation interval	44 h	44 h	44 h
Total culture period*	92 h	92 h	92 h

*Exposure started 48 h after culture initiation

The selection of the concentrations was based on data from the pre-experiment. In the main experiment I **without** and **with** metabolic activation 2500 µg/mL and 4000 µg/mL test item, respectively, and in experiment II 200 µg/mL test item was selected as the highest concentration for microscopic evaluation.

The following concentrations were evaluated for micronuclei frequencies:

Experiment I with short-term exposure (4 h):
without metabolic activation: 200, 250 and 2500 µg/mL
with metabolic activation: 500, 1000, 2000 and 4000 µg/mL

Experiment II with long-term exposure (44 h):
without metabolic activation: 125, 175 and 200 µg/mL

No precipitate of the test item was noted in the cultures at the end of treatment in any concentration group evaluated in experiment I and II.

If cytotoxicity is observed the highest concentration evaluated should not exceed the limit of 55% ± 5% cytotoxicity according to the OECD Guideline 487 (Test No. 487 2016). Higher levels of cytotoxicity may induce chromosome damage as a secondary effect of cytotoxicity. The other concentrations evaluated should exhibit intermediate and little or no toxicity. However, OECD 487 does not define the limit for discriminating between cytotoxic and non-cytotoxic effects. According to laboratory experience this limit is a value of the relative cell growth of 70% compared to the negative/solvent control which corresponds to 30% of cytostasis.

In experiment I **without** metabolic activation no increase of the cytostasis above 30% was noted up to 500 µg/mL. At 2500 µg/mL a cytostasis of 54% was observed. In experiment I **with** metabolic activation no increase of the cytostasis above 30% was noted up to 500 µg/mL. At 1000 µg/mL a cytostasis of 39%, at 2000 µg/mL a cytostasis of 49% and at 4000 µg/mL a cytostasis of 59% was observed. In experiment II **without** metabolic activation no increase of the cytostasis above 30% was noted up to 175 µg/mL. At 200 µg/mL a cytostasis of 53% was observed.

In experiment I and II **without** and **with** metabolic activation no biologically relevant increase of the micronucleus frequency was noted after treatment with the test item.

The nonparametric χ^2 Test was performed to verify the results in both experiments. No statistically significant enhancement ($p<0.05$) of cells with micronuclei was noted in the dose groups of the test item evaluated in experiment I and II **with** and **without** metabolic activation. The χ^2 Test for trend was performed to test whether there is a concentration-related increase in the micronucleated cells frequency in the experimental conditions. No statistically significant increase in the frequency of micronucleated cells under the experimental conditions of the study was observed in experiment I and II.

Methylmethanesulfonate (MMS, 25 and 65 µg/mL) and cyclophosphamide (CPA, 15 µg/mL) were used as clastogenic controls. Colchicine (Colc, 0.01 and 0.4 µg/mL) was used as aneugenic control. All induced distinct and statistically significant increases of the micronucleus frequency. This demonstrates the validity of the assay.

In conclusion, it can be stated that during the study described and under the experimental conditions reported, the test item Cellulase produced with *T. reesei* did not induce structural and/or numerical chromosomal damage in human lymphocytes.

Therefore, Cellulase produced with *T. reesei* is considered to be non-mutagenic with respect to clastogenicity and/or aneugenicity in the *in vitro* Mammalian Cell Micronucleus Test.

C.2.2.3. 90-Day Repeated Dose Oral Toxicity Study in Wistar Rats with Cellulase produced with *T. reesei*

This test was conducted in line with OECD Guidelines for Testing of Chemicals, Section 4, No. 408, "Repeated Dose 90-day Oral Toxicity Study in Rodents". The aim of this study was to assess the possible health hazards which could arise from repeated exposure of Cellulase produced with *T. reesei* via oral administration to rats over a period of 90 days.

The test item was administered daily in graduated doses to 3 groups of test animals, one dose level per group for a treatment period of 90 days. Animals of an additional control group were handled identically as the dose groups but received aqua ad injectionem (sterile water), the vehicle used in this study. The 4 groups comprised of 10 male and 10 female Wistar rats. Different doses were evaluated where there are control, low dose (LD), medium dose (MD) and high dose (HD).

The highest dose tested in the 90-day oral toxicity studies was 1000 mg TOS/kg/body weight/day.

The test item formulation was prepared at least every 10 days. The test item was dissolved in aqua ad injectionem and administered daily during a 90-day treatment period to male and female animals. Dose volumes were adjusted individually based on weekly body weight measurements.

During the period of administration, the animals were observed precisely each day for signs of toxicity. The animal that had to be sacrificed for animal welfare reasons was examined macroscopically and at the conclusion of the test, surviving animals were sacrificed and observed macroscopically.

Body weight and food consumption were measured weekly. At the conclusion of the treatment period, all animals were sacrificed and subjected to necropsy. The wet weight of a subset of tissues was taken and a set of organs/tissues was preserved.

A full histopathological evaluation of the tissues was performed on high dose and control animals. These examinations were not extended to animals of all other dosage groups. Only organs and tissues of the other dosage groups showing changes in the high dose group were examined.

Conclusion:

There was no test item-related effect observed on mortality, clinical signs, body weight development, food consumption, functional observation battery, weekly detailed clinical observations, haematology and blood coagulation, hormone analysis, clinical biochemistry, urinalysis, gross pathological findings, organ weight and histopathology in males and females sacrificed at the end of treatment period. Therefore, the histopathological NOAEL (no observed adverse effect level) could be established at 1000 mg/kg body weight.

Comments on the safety of AR-577

The original Cellulase preparation produced with *T. reesei* has been subjected to several tests as part of its safety assessment for the production of food products. In toxicological tests that have been performed, including a 90-days repeated dose rat feeding study, no toxicity was detected.

For more details on the safe strain lineage rationale on why the presented toxicological studies are applicable to AR-577 *T. reesei* fructanase strain, please refer to Appendix CCI.

It is concluded that the use of the fructanase produced with the current genetically modified *T. reesei* AR-577 as a processing aid in food processes does not pose any significant risk to human health.

Because the host organism is safe and because the genetic modifications are well characterized and specific utilizing well-known plasmids for vector constructs, and the introduced genetic material does not encode and express any toxic substances, it is concluded that the use of the fructanase from genetically

modified *T. reesei* AR-577 as a processing aid in food processes would pose no significant risk to human health.

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

A homology search was performed from the non-redundant protein sequences database using the BLAST-P. The amino acid sequence of the fructanase (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 and found in Appendix CCI it can be concluded that the fructanase 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 fructanase gene from *Lactobacillus sp. e*xpressed in *Trichoderma reesei*.

Name of the enzyme protein: Fructanase

Production strain: *Trichoderma reesei AR-577*

C.4.2. Donor

Name of the Donor: *Lactobacillus sp.*

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 fructanase, there is a possibility of causing such occupational allergy in sensitive individuals. However, the possibility of an allergic reaction

to the fructanase 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 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[7].
- 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).

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

- 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 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

To specifically evaluate the risk of the fructanase cross reacting with known allergens and induce a reaction, the sequence homology to known allergens was performed. The testing involved using an 80-amino acid (aa) sliding window search, 8-amino acid search and conventional FASTA alignment of the full-length protein sequence (overall homology), with the threshold of 35% identity as recommended by the FAO/WHO in 2001 (Food and Agriculture Organization of the United Nations January/2001) and the Codex Alimentarius in 2003 (Codex Alimentarius Commission 30 June - 5 July, 2003) for the 80mer sliding window search.

A sequence homology comparison test was performed using a database of allergens from the Food Allergy Research and Resource Program (FARRP), University of Nebraska, Allergen Database (Version 21, 14 February 2021), which contains the amino acid sequences of known and putative allergenic proteins. The amino acid sequence of the fructanase subject to this dossier was scanned using two search methods. Full details are presented in Appendix CCI.

The first method was a FASTA alignment for the full-length fructanase sequence to any allergenic proteins in the Allergen Online database. No matches having greater than 35 % identity were found from the AllergenOnline database using the full-length search. No matches having greater that 35 % identity were found from the AllergenOnline database using the 80-mer sliding window search. According to the results obtained from the alignments and taking into account the most recent scientific recommendations on the interpretation of such data lead us to conclude that the *lactobacillus sp.* Fructanase enzyme is of no allergenic 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 for the production of fructanase is the fungus *Trichoderma reesei*.

Scientific name:

Genus: *Trichoderma reesei*

Species: *Trichoderma reesei*

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 into synonymy 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.).

Taxonomic studies have shown that the species *Trichoderma reesei* consists only of this single isolate QM6a and its derivatives (e.g. Rut Series, Montenecourt and Eveleigh, 1977, 1979; QM9123 and QM9414, Mandels *et al*., 1971 – as reviewed by Nevalainen et al. (1994)).

Synonyms[8]**:** *Trichoderma longibrachiatum*

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). *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[9] in 2012, concluding that the organism will not present an unreasonable risk of injury to health or the environment when used as a recipient microorganism provided that certain criteria for the introduced genetic material and the physical containment conditions are met (submerged fermentation).
- 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[10]. 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. longibrachiatum* A83 (previously named *T. reesei* A83) and *T. longibrachiatum* QM9414

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

[9] 15 U.S.C. 2605 Toxic Substances Control Act

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

(previously named *T. reesei* 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 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.

- *T. reesei* is listed in the Natural Health Products Ingredients Database with a medicinal role as classified as a natural health product (NHP) substance falling under Schedule 1, item 1 (fungus) of the Natural Health Products Regulations. *T. reesei* is also listed as a source material for the enzyme cellulase in Health Canada's Cellulase monograph, as well as for the enzymes, beta-glucanase, hemicellulase, pectinase, and xylanases in Health Canada's Digestive Enzymes monograph (NHPID 2016).
- 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 reesei*), A1183 (Glucose oxidase from *Trichoderma reesei*), and recently 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).

Metabolites of human toxicological concern are usually produced by microorganisms for their own protection. Microbes in natural environments are affected by several and highly variable abiotic (e.g., availability of nutrients, temperature and moisture) and biotic factors (e.g., competitors and predators). Their ever-changing environments put a constant pressure on microbes as they are prompted by various environmental signals of different amplitude over time. In nature, this results in continuous adaptation of the microbes through inducing different biochemical systems; e.g. adjusting metabolic activity to current availability of nutrients and carbon source(s), or activation of stress or defence mechanisms to produce secondary metabolites as 'counter stimuli' to external signals (Klein and Paschke 2004; Earl et al. 2008). On the contrary, culture conditions of microbial production strains during industrial scale fermentation have been optimized and 'customized' to the biological requirements of the strain in question (see e.g.

review by Parekh et al. (2000). Thus, the metabolic activity and growth of a particular microbial production strain during the fermentation process (primarily the 'exponential growth phase') will focus on efficiently building cell biomass which in turn produces the molecule of interest. Industrial fermentations are run as monocultures (i.e., no external competitors or predators) with optimal abiotic conditions; and the fermentation process is terminated before or when the production strain enters the 'stationary growth phase'. Hence, there are no strong environmental signals that would induce stress (e.g., lack of nutrients or low/high temperatures) or defence mechanisms (e.g., production of antibiotic, antiviral or neurotoxic molecules). Biosynthesis of stress- and/or defence-related secondary metabolites of toxicological relevance by industrial microbial production organisms during the fermentation process is thus highly unexpected (Sanchez and Demain 2002) (Sanchez and Demain 2002) and is furthermore avoided from an economical perspective to optimize enzyme production.

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

- Under controlled industrial fermentation conditions, the organisms are not subjected to significant stress: the literature indicates that the biosynthesis of peptaibols is a defence response against other fungi when subjected to environmental stress such as the lack of nutrients (Tisch and Schmoll 2010; Komon-Zelazowska et al. 2007).
- Standard industrial fermentation process times are short for peptaibol induction: peptaibols have mostly been isolated from very old cultures of *Trichoderma*, at least 15 days of cultivation (Kubicek et al. 2007)(Kubicek et al. 2007). Industrial fermentation processes for *Trichoderma reesei* can be up to 10 days but are typically shorter (3-8 days).

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

Trichothecenes: Trichothecenes constitute a very large family of chemically related mycotoxins, which are cytotoxic and immune suppressive and produced by various species. Based on contamination of grains with *Fusarium* species, trichothecenes are known toxins occurring occasionally in food and feed.

Only in one publication has *Trichoderma reesei* been suggested to produce the trichothecene Trichodermin (Watts et al. 1988). However, in this publication, the identity of the *Trichoderma reesei* strain is doubted. The work of Watts refers to Godtfredsen and Vangedal (1965) in which Trichodermin was identified from a culture of *Trichoderma viride*. Before 1969, it was believed that all *Trichoderma* isolates belonged to one single species, and all were called as *Trichoderma viride*. Later on, *Trichoderma viride* producing cellulase (derived from QM6a) was recognised as a separate species and re-named *Trichoderma reesei*. It is therefore highly probable that there was some confusion in determining the identity of those species. In addition the methodology used in Watts et al. (1988) to identify Trichodermin is not conclusive.

Moreover, it has been recently shown that the gene for the synthesis of Trichodermin is absent from *T. reesei* genome: a blastp analysis using TRI5 from *Trichoderma brevicompactum* (a strain producing trichothecenes) results in no hits on *T. reesei* genome sequence. This indicates that *T. reesei* is unable to initiate trichothecene biosynthesis (Cardoza et al. 2011). Based on the information given above the relevance of Trichodermin for toxicity of *Trichoderma reesei* products can be excluded.

Based on the information given above the relevance of Trichodermin for toxicity of *Trichoderma reesei* products can be excluded.

It is relevant to note that during recent years, genetic engineering techniques have extensively been used to improve the industrial production strains of *T. reesei*, and in addition, considerable experience of safe use of recombinant *T. reesei* strains in industrial scale has accumulated. Furthermore, food enzymes from

Trichoderma reesei have been subjected to several trials as part of their safety assessment for the use in food products manufacturing processes including 90-day toxicological tests.

The safety of the fructanase produced by the genetically modified *Trichoderma reesei* AR-577 from a toxicological perspective is supported by the historical safety of strain lineage. Toxicological studies were performed on a strain (*Trichoderma reesei* AR-852) which derives from the same intermediate strain within the strain lineage of *Trichoderma reesei* AR-577. Toxicological tests that have been performed included a 90-day repeated dose study.

These results show that there is no need for any toxicological concern with fermentation products as produced by use of *Trichoderma reesei*. Based on the available data, it is concluded that the organism *T. reesei* is non-pathogenic and non-toxigenic and *T. reesei* AR-577 is safe to use as the production organism for fructanase enzyme preparation (please refer to SSL description).

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 polygalacturonase) used *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/NZ	Cellulase Glucan 1-3 beta-glucosidase	Australia New Zealand Food Standards Code – Schedule 18 – Processing aids (legislation.gov.au)

	Beta-glucanase Hemicellulase complex Gluco-amylase Endo 1,4-beta xylanase Serine Endopeptidase	
Canada	Cellulase Glucanase Pentosanase Xylanase Protease	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
USA[11]	Pectin lyase Transglucosidase Glucoamylase Phospholipase A2 Pectin esterase Mannanase Endo-1,4-beta xylanase 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 558 GRAS Notice Inventory, GRN 566 GRAS Notice Inventory, GRN 628

[11] GRAS affirmations and GRAS notifications

Presence of introduced genes that encode microbial resistance

The review article by Nevalainen et al. (1994) reveals that some species belonging to *Trichoderma* genus are able to secrete various types of antibiotics in laboratory cultures. However, strains of *T. reesei* used in industrial applications are proven to be absent of antibiotic activities (Hjortkjaer et al. 1986; Coenen et al. 1995). The absence of antibiotic activities, according to the specifications recommended by JECFA (JECFA, 2006), was also confirmed from four AR-577 production batches. The analyzed data are presented in **Table 3** and Appendix CCI.

Additionally, no genes have been introduced that encode antimicrobial resistance to the parental or recipient organisms.

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 and the recipient strain described here and its genetic modifications have been used since 1994.

During recent years, 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. From above, secondary metabolites are of no safety concern in fermentation products derived from *Trichoderma reesei*. 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 other safe gene products.

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

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

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

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

This section contains summarized information. The detailed information is provided in the Appendix CCI.

E.2. Host/recipient organism

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

E.3. Donor

The *Trichoderma reesei* host strain is genetically modified with a fructanase gene deriving from *Lactobacillus sp.*

E.4. Genetic modification

The *Trichoderma reesei* AR-577 strain was constructed for *Lactobacillus sp.* fructanase production. In constructing the strain AR-577, the expression cassette (fructanase gene under the control of the *T. reesei* promoter) was introduced into the genome of the *Trichoderma reesei* recipient strain.

The transformation of the recipient *T. reesei* strain with the expression cassette was performed as described in Penttilä et al. (1987) with modifications. The transformants were selected according to their ability to grow on acetamidase plates (*amdS* marker gene). No vector DNA was expected to be included in the DNA preparation used for transformation. The expression cassette and genetic modification process are described in Appendix CCI.

E.5. Stability of the Transformed Genetic Sequence

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

The stability and potential for transfer of genetic material was assessed as a component of the safety evaluation of the production microorganism. Southern blot analyses were performed to the genome of the *T. reesei* production strain AR-577. Results indicated that several copies of the expression cassettes were integrated in the genome of strain AR-577 and that the production strain is stable in terms of genetic traits. For more details, please see Appendix CCI.

Demonstration of the absence of the GMM in the product

The absence of the GMM in the final enzyme preparation of AR-577 is achieved through filtering after the fermentation process. All viable cells of the production strain AR-577 are removed during the down-stream processing: the fermentation broth is filtered through a pressure filter, concentrated by ultrafiltration (nominal molecular weight cut-off 10000 Da), and finally filtered with sheet filters. The procedures are completed by trained staff based on documented standard operating procedures complying with the requirements of the quality system.

The fructanase food enzyme preparation is free of detectable, viable production organism. The absence of the production strain is confirmed for every production batch. Three different samples were analyzed for absence of the production strain as summarized in **Table 3**. Absence of the production strain in the final product is confirmed by a Roal[12] in-house method, which is validated in-house and company specific. The method document is provided in this submission as Appendix CCI.

[12] Roal Oy is the sole manufacturer of AB Enzymes' enzyme preparations. Roal Oy is based in Finland.

Table 3: Chemical Composition Analysis of 3 different samples of Fructanase

Batch	Liquid Enzyme P210025	Liquid Enzyme P220007	Liquid Enzyme B220086
Activity (FRU/g)	2908	3415	2863
Antimicrobial activity	Not detected	Not detected	Not detected
Presence of production	Not detected	Not detected	Not detected
***Escherichia coli* (/25 g)**	Not detected	Not detected	Not detected
***Salmonella* (/25 g)**	Not detected	Not detected	Not detected
Total coliforms (cfu*/g)	<30	<30	<30
***B. cereus* (cfu*/g)**	-	-	-
Pb (mg/kg)	< 0.05	< 0.05	< 0.05
As (mg/kg)	< 0.5	< 0.5	< 0.5
Hg (mg/kg)	< 0.05	< 0.05	< 0.05
Cd (mg/kg)	< 0.03	< 0.03	< 0.03

Inactivation of the GMM and evaluation of the presence of remaining physically intact cells

The AR-577 enzyme preparation is free from detectable, viable production organism as demonstrated in **Table 3**. As the absence of the production strain is confirmed for every production batch, no additional information regarding the inactivation of the GMM cells is required.

Information on possible presence of recombinant DNA

The *Trichoderma reesei* AR-577 enzyme preparation is produced by an aerobic submerged microbial fermentation using a genetically modified *Trichoderma reesei* strain. All viable cells of the production strain, AR-577, are removed during the down-stream processing: the fermentation broth is filtered with pressure filters and subsequent sheet filters, concentrated by ultra-filtration, optionally followed by sheet filtration(s).

After this, the final product does not contain any detectable bacterial colony forming units or recombinant DNA. Two separate food enzymes were tested for the presence of recombinant DNA using highly sensitive

and specific PCR techniques. No recombinant DNA (recDNA) of the production strain was shown to be present above the detection limits (Appendix CCI).

Absence of antibiotic genes and toxic compounds

As mentioned above, the inserted DNA does not contain any antibiotic resistance genes. Furthermore, the production of known toxins according to the specifications elaborated both in Compendium of Food Additive Specifications by the Joint FAO/WHO Expert Committee on Food Additives (FAO/WHO 2006) and the JECFA specifications for enzyme preparations[13] have been also tested for the fermentation products. Adherence to specifications of microbial counts is routinely analyzed. Three production batches produced by the production strain *Trichoderma reesei* AR-577 (concentrates) were analyzed and no antibiotic or toxic compounds were detected (**Table 3** and detailed analysis presented in Appendix CCI). The production strain does not contain any functional or transmissible antibiotic resistance genes.

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

Fructanase can be used in the manufacturing of bakery products such as, but not limited to bread, steamed bread, bread buns, tortillas, cakes, pancakes, and waffles.

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

Commercial food enzyme preparations are generally used following the *Quantum Satis* (QS) principle, i.e., at a level not higher than the necessary dosage to achieve the desired enzymatic reaction, according to Good Manufacturing Practice. The amount of enzyme activity added to the raw material by the individual food manufacturer must be determined case by case, based on the desired effect and process conditions.

[13] General Specifications and Considerations for Enzyme Preparations (fao.org)

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 **fructanase** 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 as such do not give an indication of 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) [14] (FAO/WHO 2006). 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 below.

The table below shows the range of recommended use levels for each application where the fructanase is used:

Food Application	Raw material (RM)	Suggested recommended use levels (mg TOS/kg RM)
Baking	Flour	12

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

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

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

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

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

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

Consumption of food patterns:

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

To determine the TMDI of fructanse 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 on the next page), All the TOS is assumed to be in the final product.

Applications		Raw Material	Maximum 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	Flour	12	Baked products	0.71	8.52

*<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 TMDI 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 will consequently be calculated as follows:

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)
8.52 x 0.0125 = 0.107	0 x 0.025 = 0	**0.107**

The Total TMDI is based on conservative assumptions and represents a highly exaggerated value as per the following

- It is assumed that ALL producers of the above-mentioned foodstuffs (and beverages) use specific fructanase enzyme from *Trichoderma reesei* AR-577;

- 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).

The value for the Total TMDI is highly exaggerated. Consequently, there are no safety reasons for laying down maximum levels of use.

Margin of Exposure (MoE)

According to the Safe Strain Lineage concept, the MoE for human consumption can be calculated by dividing the NOAEL by the Total Theoretical Maximal Daily Intake (TMDI). In the case of the safe strain lineage concept for *Trichoderma reesei* AR-577, there is no NOAEL. However, the NOAEL of 1,000 mg/kg body weight/day from the 90-day toxicological study from the closely related production strain *Trichoderma reesei* AR-852 is used to calculate the MoE and support the safety of the fructanase from *Trichoderma reesei* AR-577.

MoE = 1,000 mg/kg body weight/day ÷ 0.107 mg TOS/kg body weight/day = **9,390**

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

Conclusion:

To conclude, the use of the food enzyme fructanase from *Trichoderma reesei* AR-577 used as a processing aid in theproduction 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 fructanase 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.

V. List of Appendices

Section 3.1

1.1 Customer Support Letter
1.2 Formal Request for Confidential Information (CCI)
1.3a Statutory Declaration Australia
1.3b Statutory Declaration New Zealand
1.4 Checklist 3.1 and 3.3

Section 3.2

1. Product Data Sheet – VERON® FR
2. Quality Certificates
3. Safe Strain Lineage
4. Manufacturing Flow-chart

Appendix CCI – Treated as confidential information

I. Publication bibliography

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