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

AB Enzymes GmbH

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II. EXECUTIVE SUMMARY

The present application seeks to amend Standard 1.3.3. - Processing Aids of the Australia New Zealand Food Standards Code (the Code) to approve a xylanase enzyme preparation from *Trichoderma reesei* produced by AB Enzymes GmbH.

Proposed change to Standard 1.3.3 - Processing Aids

The table to clause 17, Permitted enzymes of Microbial Origin, is proposed to be amended to include a genetically modified strain of *Trichoderma reesei* as permitted source for Endo-1,4 (3)-β-xylanase (E.C. 3.2.1.8).

This application is submitted under a general assessment procedure.

Description of Enzyme Preparation

The food enzyme is a biological isolate of variable composition, containing the enzyme protein, as well as organic and inorganic material derived from the microorganism and fermentation process.

The main activity of the food enzyme is endo-1,4-ß-xylanase (IUB 3.2.1.8). The food enzyme catalyses the hydrolysis of xylosidic linkages in an arabinoxylan backbone (and other ß-1,4-linked xylans) resulting in depolymerisation of the arabinoxylan into smaller oligosaccharides.

It uses xylans as substrate. Xylans are constituents of hemicellulose, a structural component of plant cell walls. Arabinoxylans (also known as pentosans) are highly branched xylans that occur in wheat and rye flour. Consequently, the substrate for endo-1, 4-β-xylanase occurs naturally in vegetable based foods and can be found in various plant materials including the cell walls and endosperm of cereals, such as wheat and barley.

Apart from endo-1,4-ß-xylanase, the food enzyme also contains other enzymatic side activities in small amount, which are typical to the production organism *Trichoderma reesei*. Those include ß-glucanase

and cellulase. Adverse effects from side activities of the food enzyme are not considered a concern, due to small amounts and the fact that such enzyme activities have been used and approved for decades in food processing.

The enzyme is produced by submerged fermentation of a genetically modified *Trichoderma reesei* with a xylanase gene from *Thermopolyspora flexuosa* (previously named *Nonomuraea flexuosa*).

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

Use of the Enzyme

The food enzyme object of the dossier is typically used in baking process and processes of other cereal based products (such as pasta and noodles), starch processing, distilling, and brewing.

In principle, the enzymatic conversion of (arabino)xylans with the help of endo-1,4-β-xylanase can be used in the processing of all food raw materials which naturally contain(arabino)xylans.

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

Benefits

This dossier is specifically submitted for the use of endo-1,4-β-xylanase in baking processes and other cereal based processes, brewing, grain processing and potable alcohol production. Below, the benefits of the use of industrial endo-1,4-β-xylanase in those processes are described. The beneficial effects are of value to the food chain because they lead to better and/or more consistent product quality. Moreover, the applications lead to more effective production processes, resulting in better production

economy and environmental benefits such as the use of less raw materials and the production of less waste.

Baking processes:

Endo-1,4-β-xylanase can be used in the manufacturing of bakery products such as, but not limited to, bread, biscuits, steamed bread, cakes, pancakes, tortillas, wafers and waffles.

Arabinoxylans provide functional properties during bread making due to their ability to interact with gluten, bind water and provide dough viscosity. Limited hydrolysis of the water-unextractable arabinoxylans with the help of endo-1,4-β-xylanase results in solubilized arabinoxylans with lower molecular weights, which improves the functional baking properties of these polysaccharides.

The benefits of the conversion of arabinoxylans with the help of endo-1,4-β-xylanase in baking are:

- Facilitate the handling of the dough (improved extensibility and stability, reduced stickiness leading to reduced losses of dough)
- Improve the dough's structure and behaviour during the baking step
- Ensure a uniform and slightly increased volume and an improved crumb structure of the bakery product, which might otherwise be impaired by processing of the dough
- Reduce batter viscosity, beneficial in the production process for e.g. waffles, pancakes and biscuits

Endo-1,4-β-xylanase can also be used in the processing of other cereal based products such as, but not limited to, pasta, noodles and snacks, where they can improve the dough processability and accelerate the drying step, thereby shortening the process time. Arabinoxylans provide functional properties during pasta, noodle and snack making due to their ability to interact with gluten, bind water and provide dough viscosity. Limited hydrolysis of arabinoxylans with the help of endo-1,4-β-xylanase improves the functional properties of these polysaccharides.

The benefits of the action of endo-1,4-β-xylanase are:

- Facilitate the handling of the dough
- Increase firmness and reduce oil absorption in instant noodles
- Reduce checking (formation of hair line cracks)
- Accelerate the drying step, thereby shortening the process time.

Furthermore, endo-1,4-β-xylanase has been used in baking and other cereal based products for over 25 years (Beg et al. 2001).

Brewing and other cereal base beverages:

During beer production, the xylans present in the cell walls of the grain are partly responsible for wort and beer viscosity - which impairs wort (lautering or mash filtration) and beer filtration. The benefits of the conversion of (arabino)xylans with the help of xylanase in brewing are:

- Decreased wort viscosity
- Faster and more predictable lautering or mash filtration
- Faster and better beer filterability
- Improved extraction yield
- Reduced consumption of beer filtration aids

Grain processing

Cereals are highly complex structures causing technical difficulties during processing when milled and when fractionated to starch, gluten and fibres. Enzyme systems that act on the cereal components, including xylans, are used to ensure smooth and efficient processing, facilitate the separation (by opening the grain structure) and ensure high quality of the polysaccharide and gluten fractions.
Grain processing also covers milling and peeling. Insufficiently hydrolysed grain cell wall components reduce the effectiveness of the mechanical treatments such as milling and peeling.

The benefits of the conversion of (arabino)xylans with the help of xylanase in Grain processing are:

- Reduced viscosity of the wheat flour batter, facilitating gluten and starch separation

- Improved gluten and starch purity due to greater extraction yield of the high value fraction and efficient removal of arabinoxylan
- Energy savings due to less use of process water, lower evaporator costs and decreased production time.
- Degradation of cell wall components increasing effectiveness of the mechanical treatments such as milling and peeling.

Xylanase is typically added in grain processing during the initial steps such as conditioning, homogenization and dough preparation. The result of the grain processing is food ingredients such as flour or cereal fractions such as starch, gluten, fiber. Xylanase is not necessarily inactivated during grain processing process, but the resultant food ingredients (separated fractions) are further used in other food processes where the enzyme will be inactivated.

Use of the fractions obtained after grain processing:

Flour is used as a food ingredient in baking process. The starch fraction might be used as a food ingredient in other food applications such as baking and dairy, or for technical applications (e.g. for paper production), and for ethanol production or alternatively as animal feed. Starch might also be processed into glucose, maltose high fructose and other syrups which are themselves used in a number of food products.

The fibre fraction is used in baking as well as for animal feed.

The gluten fraction is mostly used in baking to improve the properties of the flour. Gluten might also be used in other food applications such as meat processing.

Alcohol Production

In Potable alcohol production the high levels of xylans, cellulose, lichenin and beta-D-glucans results in high viscosity due to the water-binding capacity. High viscosity has negative effects on alcohol

production because it limits solid concentration in mashing and reduces efficiency in the mixing, separation and filtration processes.

Xylanase is used in distilling industrial applications prior to the liquefaction of highly concentrated mashes. The benefits of the conversion of (arabino)xylans with the help of xylanase in potable alcohol production are:

- Decrease viscosity of grain mashes
- Better processing (solid/liquid separation, resulting in higher solid concentration during mashing; increase fermentable sugars and improve mass transfer during fermentation)
- Reduce fouling in the heat exchangers and distilling equipment
- Increase flexibility in the choice of raw materials and allow to use more grain and less water
- Potential higher alcohol (ethanol) yield as result of better processing, and thereby less use of raw materials.
- Reduce fuel consumption due to better heat transfer

Safety Evaluation

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

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

The product is free of production strain and recombinant DNA.

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

- **France**: The enzyme preparation was safety assessed according to the Guidelines for the evaluation of food enzymes. This resulted in the authorisation of the enzyme product by the French authorities.
- **USA**: A GRAS determination was done and notified to the US FDA in December 2015 (GRN000628). In the reply letter from FDA dated September 14th, 2016, the agency has no questions regarding AB Enzymes' determination that the xylanase enzyme reparation is GRAS for its intended use.
- **EFSA/ EU Commission**: a dossier was submitted in 2014 in compliance with Regulation (EC) 1332/2008 and is currently being reviewed by EFSA. In addition, the same enzyme has also been approved in the EU for the use in feed.

Conclusion

Based on the safety evaluation, AB Enzymes GmbH respectfully request the inclusion of *Trichoderma reesei* expressing a xylanase gene from *Thermopolyspora flexuosa* (previously named *Nonomuraea flexuosa*) in the table to clause 17 of standard 1.3.3.; Permitted enzymes of Microbial Enzymes.

III. INTRODUCTION

The dossier herein describes a *Trichoderma reesei* produced xylanase (RF5427) expressing a gene from *Thermopolyspora flexuosa* (previously named *Nonomuraea flexuosa*) produced by submerged fermentation.

This dossier is specifically submitted for use of endo-1,4-β-xylanase used in the manufacturing of bakery products such as, but not limited to, bread, biscuits, steamed bread, cakes, pancakes, tortillas, wafers and waffles, brewing, grain processing, and potable alcohol production. A further description of the enzyme in these food technology applications will be given in subsequent sections.

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 endo-1,4-β-xylanase are described. Information on the mode of action, applications, and use levels of endo-1,4-β-xylanase and enzyme residues in final food products are described. The safety studies outlined herein indicate that the endo-1,4-β-xylanase enzyme preparation from *T. reesei* shows no evidence of pathogenic or toxic effects. Estimates of human consumption and an evaluation of dietary exposure are also included.

IV. Section 3.1, GENERAL REQUIREMENTS

3.1.1. Executive Summary

An Executive Summary is provided as a separate copy together with this application. The executive summary must be provided as an electronic file separate from other parts of the application.

3.1.2. Applicant Details

Applicant's name

Company
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Germany

Telephone Number

Email Address

Nature of Applicant's Business
Biotechnology

Dossier prepared by

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Germany

3.1.3. Purpose of the Application

To amend the table to clause 17, Permitted enzymes of Microbial Origin, is proposed to be amended to include a genetically modified strain of *Trichoderma reesei* as permitted source for Endo-1,4 (3)-β-xylanase (E.C. 3.2.1.8).

3.1.4. Justification for the Application

The need for the proposed change:

Trichoderma reesei expressing a xylanase gene from *Thermopolyspora flexuosa* (previously named *Nonomuraea flexuosa*) is not present as an approved source in the table to clause 17 of standard 1.3.3.; Permitted enzymes of Microbial Enzymes. AB Enzymes GmbH is requesting that this source organism be added.

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

In principle, the enzymatic conversion of (arabino) xylans with the help of endo-1,4 β-xylanase can be of benefit in the processing of all foods and food ingredients which naturally contain the substrate.

This xylanase enzyme developed by AB Enzymes GmbH has specific benefits to food manufacturing, such as viscosity reduction and separation depending on substrate and process conditions, facilitate the handling of dough, improve dough structure, increase firmness and reduce oil absorption in instant noodles, etc. The enzyme is one of AB Enzymes latest achievements and has showed great potential in food manufacturing as detailed in this customer support letter, Appendix #1.1.

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

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

3.1.6. Regulatory Impact Statement:

The addition of the enzyme to Standard 1.3.3 is not intended to place any costs or regulatory restrictions on industry or consumers. Inclusion of the enzyme will provide food manufacturers with an alternate choice to help improve baking and other cereal based products, grain processing, brewing and distilling. For government, the burden is limited to necessary activities for a variation of Standard 1.3.3.

3.1.7. Impact on International Trade:

This change is not expected to have an impact on international trade.

3.1.8. Information to Support the Application

Public Health and Safety Issues related to the Proposed Change:

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

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

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

The product is free of production strain and recombinant DNA.

Consumer choice related to the Proposed Change:

Consumer choice is not expected to be changed directly as the enzyme is used as a processing aid and is not purchased by consumers. Endo 1,4-β-xylanase 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 endo 1,4-β-xylanase do not have other characteristics than what is expected by the consumer. Consumers could be impacted indirectly by companies able to pass cost savings from utilizing enzymes in food processing on to their customers.

3.1.9. Assessment Procedure

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

3.1.10. Confidential Commercial Information (CCI)

Detailed information on the construction and characteristics of the genetically modified production strain is provided in the confidential Appendix 14. A summary of this information is given in section E of section 3.2.2. The formal request for treatment of Appendix 14 as confidential commercial information (CCI) is included as Appendix #1.2.

3.1.11. Other Confidential Information

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

3.1.12. Exclusive Capturable Commercial Benefit (ECCB)

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

3.1.13. International and other National Standards

International Standards:

Use of enzymes as processing aids for baking, distilling, brewing and grain processing is not restricted by any Codex Alimentarius Commission (Codex) Standards.

National Standards:

Use of enzymes as processing aids in food applications (baking, brewing etc.) has specific standard in France (arrêté du 19 octobre 2006[1]), and the use of this enzyme has been approved for the accordant food applications in this dossier (please see **Section 3.3)**. Canada also has food standards for white bread, alcoholic beverages, and grain and bakery products. Please note that this enzyme has not yet been submitted to Health Canada as of yet.

3.1.14. Statutory Declaration

The Statutory Declaration is included as Appendix #1.4.

3.1.15. Checklist

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 September 1st 2013, are the following sections:

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

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

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

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

A. Technical Information of the Processing aid

A.1. Information on the type of processing aid

This dossier includes a xylanase enzyme, produced with the help of *Trichoderma reesei* strain RF5427. The representative current commercial products are Veron® HTX and Rohalase® SEP Visco.

Xylanase is a microbial produced enzyme and already belongs to the table to clause 17 of standard 1.3.3.; Permitted enzymes of Microbial Enzymes.

Enzyme preparations are generally used in *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 endo-1,4-β-xylanase used in the manufacturing of bakery products (such as, but not limited to, bread, biscuits, steamed bread, cakes, pancakes, tortillas, wafers and waffles), brewing, grain processing, and potable alcohol production. 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	Endo-1,4-ß-xylanase
Common names	Endo-1,4 (3)-beta-xylanase; endo-(1→4)-β-xylan 4-xylanohydrolase; endo-1,4-xylanase; xylanase; β-1,4-xylanase; endo-1,4-xylanase; endo-β-1,4-xylanase; endo-1,4-β-D-xylanase; 1,4-β-xylan xylanohydrolase; β-xylanase; β-1,4-xylan xylanohydrolase; endo-1,4-β-xylanase; β-D-xylanase
IUBMB No.	EC 3.2.1.8
CAS number	9025-57-4

A.2.2.Enzyme Preparation

The commercial names of representative enzyme preparations (RF5427) are: Rohalase® SEP Visco and Veron® HTX. The product data sheets are provided in Appendix #1.

A.2.3.Enzyme preparation compositions:

Composition Rohalase® SEP Visco (liquid)	
Xylanase Concentrate	22.9%
Sorbitol	45.0%
Tri-sodium, citrate dehydrate	1.5%
Citric acid, anhydrous	1.1%
Sodium benzoate	0.35%
Water	29.15%

Composition Veron® HTX (powder)	
Xylanase Concentrate	4.2%
Sun flower Oil	0.4%
Wheat Flour	95.4%

The main enzymatic activity of *T. reesei* RF5427 enzyme preparation is endo-1,4-ß-xylanase. This food enzyme catalyses the hydrolysis of xylosidic linkages in an arabinoxylan backbone (and other ß-1,4-linked xylans) resulting in depolymerisation of the arabinoxylan into smaller oligosaccharides.

Xylans are constituents of hemicellulose, a structural component of plant cell walls. Arabinoxylans (also known as pentosans) are highly branched xylans that occur e.g. in wheat and rye flour. Consequently,

the substrate for endo-1, 4-β-xylanase occurs naturally in vegetable based foods and can be found in various plant materials including the cell walls and endosperm of cereals, such as wheat and barley.

The methods to analyse the activity of the enzyme is company specific and is capable of quantifying endo-1,4,-β-xylanase activity as defined by its IUBMB classification. The enzyme activity of the xylanase produced by RF5427 was usually given in BXU unit (1 BXU unit is equivalent to the amount of enzyme that products, under standard conditions (pH 5.3, 50°C), one nmol of reducing sugar, i.e. xylose, from birch xylan in one second). As international units (IU), one BXU is equivalent to 0.06 micromol/minute. However, a new enzyme unit, namely TXU, was recently introduced and used for the latest developed product Rohalase® SEP VISCO. The principle is basically the same but conditions for pH and temp have been slightly adapted (Appendix #2, listed as "other" confidential information).

A.2.4.Genetic Modification

The enzyme is from a *Trichoderma reesei* host strain genetically modified with a xylanase gene deriving from *Thermopolyspora flexuosa* (previously named *Nonomuraea flexuosa*). The enzyme is not protein engineered.

Name of the enzyme protein:	**Endo-1,4-ß-xylanase**
Donor:	*Thermopolyspora flexuosa*
Host:	*Trichoderma reesei*
Production strain:	*Trichoderma reesei* RF5427

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

Rohalase® SEP Visco:

Properties	
pH Value	4.1-4.5
Density	1.1-1.15

Appearance	Clear brown Liquid
Odour	Typical

Veron® HTX:

Properties	
pH Value	4.1-4.5
Density	1.1-1.15
Appearance	Powder
Odour	light beige coloured with aromatic smell

The food enzyme catalyses the hydrolysis of arabinoxylan and other ß-1,4-linked xylans into arabinoxylan-oligosaccharides as well as shorter chain length arabinoxylan-polysaccharides. Such enzyme activity is widely present in nature and in particular in food ingredients. The substrates and the reaction products are themselves present in food ingredients. No reaction products which could not be considered normal constituents of the diet are formed during the production or storage of the enzyme treated food. Consequently, no adverse effect on nutrients is expected.

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

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

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

A.4. Manufacturing Process

Like all food enzymes, endo-1,4,-β-xylanase described in this dossier is manufactured in accordance with current Good Manufacturing Practices for Food (cGMPs) and the principals of Hazard Analysis of Critical Control Points (HACCP). Compliance to Food Hygiene Regulation is regularly controlled by relevant food inspection services in Finland. Quality certificates are provided in Appendix #3.

T. reesei RF5427 endo-1,4,-β-xylanase described herein is produced by controlled submerged fermentation. The production process involves the fermentation process, recovery (downstream processing) and formulation and packaging. A manufacturing flow-chart is given in Appendix #4.

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

A.4.1.Fermentation

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

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

- Inoculum
- Seed fermentation
- Main fermentation

A.4.2.Raw materials

The raw materials used in the fermentation and recovery processes are standard ingredients that meet predefined quality standards controlled by Quality Assurance for ROAL Oy. The safety is further

confirmed by toxicology studies (See **Section C**). The raw materials conform to either specifications set out in the Food Chemical Codex, 10th edition, 2016 or The Council Regulation 93/315/EEC, setting the basic principles of EU legislation on contaminants and food, and Commission Regulation (EC) No 1881/2006 setting maximum limits for certain contaminants in food. The raw materials used for the formulation are of food grade quality.

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

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

A.4.4. Inoculum

A suspension of a pure culture of *T. reesei* RF5427 is aseptically transferred to a shake flask (1 liter) containing fermentation medium.

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

A.4.5. Seed fermentation

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

A.4.6.Main fermentation

Biosynthesis of the endo-1,4,-β-xylanase product by the production strain *T. reesei* RF5427 occurs during the main fermentation.

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

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

A.4.7.Recovery

The purpose of the recovery process is:

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

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

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

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

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

A.4.8.Materials

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

- Flocculants
- Filter aids
- pH adjustment agents

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

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.

A.4.10.Primary solid/liquid separation

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

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

A.4.11.Concentration

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

A.4.12.Polish and germ filtration

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

A.4.13.Formulation and Packaging

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

For the manufacture of dry food enzyme preparations, the food enzyme is typically spray dried. The dried food enzyme is then standardized to the desired/ declared activity with food grade ingredients.
In order to reduce the dust formation for health and safety purposes, dust-binding agents (food grade) are being added during manufacturing of final products.

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

A.5. Specification for the purity and identity

The final enzyme product complies with the recommended General Specifications for Enzyme Preparations Used in Food Processing Joint FAO/WHO Expert Committee on Food Additives,

Compendium of Food Additive Specifications, FAO Food and Nutrition Paper *(Food and Agriculture Organization of the United Nations 2006)* and the Monograph "Enzyme Preparations" Food Chemicals Codex (FCC) 10th edition (2016) for food-grade enzymes. Specifications for the food enzyme preparation have been defined as follows:

Analytical data is provided in Appendix #5.

The methods used are provided in Appendix #6.

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

A.6. Analytical method for detection

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

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

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

C. Information related to the safety of an enzyme processing aid

C.1. General information on the use of the enzyme as a food processing aid in other countries

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

- **France**: The enzyme preparation was safety assessed according to the Guidelines for the evaluation of food enzymes (EFSA GL, 2009). This resulted in the authorisation of the enzyme product by the French authorities. The approval letters from the French authorities and the ANSES scientific opinions are included in Appendix #7.
- **USA**: A GRAS determination was notified to the US FDA in December 2015 (GRN000628, Appendix #8). In the reply letter from FDA dated September 14th, 2016, the agency has no questions regarding AB Enzymes' determination that the xylanase enzyme reparation is GRAS for its intended use.

- **EFSA/ EU Commission**: a dossier was submitted in 2014 in compliance with Regulation (EC) 1332/2008 and is currently being reviewed by EFSA. In addition, the same enzyme has also been approved in the EU for the use in feed with commercial name Econase XT (See Appendix #9 - Commission Regulation (EC) No 902/2009).

C.2. Information on the Potential Toxicity of the Enzyme Processing Aid

C.2.1.Information on the enzyme's prior history of human consumption and its similarity to proteins with a history of safe human consumption

As documented below, xylanases from various micro-organisms (including genetically modified ones) are widely accepted for their use in several applications such as the preparation of fruit juices, beer, and grain and baking processing. See accordant table below:

Non-exhaustive list of authorisations of authorised xylanases from production organisms other than *Trichoderma reesei*		
Authority	**Production organism**	**Reference**
JECFA	*Bacillus subtilis* *Thermomyces lanuginosus expressed in Fusarium venenatum* *Humicola insolens (mixed xylanase and beta-glucanase*	**JECFA Evaluations, 2004** **JECFA 52, 2004** **JECFA 52, 2004**
Australia/ New Zealand	*Humiloca insolens* *Aspergillus niger*	**Standard 1.3.3 processing aids**
Canada	*Aspergillus oryzae* *Bacillus subtilis* *B. subtilis (as hemicellulase, pentosanase)*	**B.16.100, Table V**
USA[3]	*Fusarium venenatum*	**GRAS Notice Inventory, GRN 54**

	Humicola insolens *Talaromyces emersonii*	**GRAS Notice Inventory, GRN 195** **GRAS Notice Inventory, GRN 479**
France	*A. niger (endo xylanase)*	**Arrêté du 19 octobre 2006 relatif à l'emploi d'auxiliaires technologiques dans la fabrication de certaines denrées alimentaires | Legifrance**
	A. niger (as hemicellulase)	
	B. subtilis (as hemicellulase)	
	B. subtilis (as xylanase)	
	Humicola insolens (as pentosanase)	
	A. oryzae	

The endo-1,4-β-xylanase enzyme preparation from *T. reesei* RF5427, expressing the recombinant gene (xylanase) deriving from *Thermopolyspora flexuosa* (*Nonomuraea flexuosa)* was evaluated according to the Pariza and Johnson Decision Tree. The decision tree is based on the safety evaluation published by Pariza and Foster in 2001, adapted from their original evaluation in 1983. Based on the Pariza and Johnson decision tree analysis, AB Enzymes concludes that the endo-1,4-β-xylanase enzyme preparation is safe, see Appendix #10.

C.2.2.Toxicological Studies

This section describes the studies performed to evaluate the safety of the RF5427 endo-1,4-β-xylanase enzyme preparation. All safety studies were performed according to internationally accepted guidelines (OECD or FDA) and are in compliance with the principles of Good Laboratory Practice (GLP) according to the FDA/OECD.

The safety of the endo-1,4-β-xylanase enzyme product was already evaluated by the FEEDAP and GMO Panels (Panel on Additives and Products or Substances used in Animal Feed and Panel on Genetically Modified Organisms) on behalf of the European Commission. The Panels concluded that the endo-1,4-β-xylanase enzyme preparation of *Trichoderma reesei* CBS114044 (same as the one we use for the production of the food enzyme object of this dossier) can safely be used in animal feed and the

Commission Regulation (EC) No 902/2009[2] has been published on 28 September 2009 authorising the enzyme preparation as a feed additive for weaned piglets, chickens for fattening, chickens reared for laying, turkeys for fattening and turkeys reared for breeding.

It is generally accepted that known commercial enzyme preparations of *T. reesei* are not toxic and since endo-1,4-β-xylanase is a natural constituent in the environment, it is concluded that the endo-1,4-β-xylanase enzyme from *T. reesei* RF5427 is safe as for use as a food processing aid in various applications.

To further confirm that the endo-1,4-β-xylanase enzyme preparation does not have any toxic properties and to ensure the toxicological safety of the use of the enzyme preparation from *T. reesei*, the following studies were conducted:

- Ames test – Appendix #11
- Chromosomal aberration test, in vitro – Appendix #12
- 90 Day Oral Toxicity Study (Rodents) – Appendix #13

Bacterial Reverse Mutation Test

Endo-1,4-β-xylanase enzyme preparation (approx. 94% TOS) was tested for mutagenic activity in *Salmonella typhimurium* strains TA 1535, TA 1537, Ta 98 and TA 100 and E*scherichia coli* WP2uvrA at concentrations ranging from 17 to 5000 µg/ml.

The test, based on OECD Guidelines No. 471 the European Commission Annex V Test Method B13 and B14, ICH guidelines CPMP/ICH/141/95 and CPMP/ICH/174/95 and USA EPA 712-C-98-247, was run at Charles River Laboratories, Edinburg, UK during 28 April – 23 July 2004.

There was no toxicity to the bacteria and no precipitation of the test item was observed in either mutation assay, in either the presence or the absence of a metabolizing enzyme mixture (S9 mix).

[2] http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32009R0902

It was concluded that endo-1,4-β-xylanase was not mutagenic to *Salmonella typhimurium* or *E. coli* when tested in sterile, ultra-pure water up to a predetermined limit of 5000 µg/ml.

Chromosomal Aberration Test

Endo-1,4-β-xylanase enzyme material (approx. 94% TOS) was tested for clastogenic activity, with duplicate, Chinese hamster ovary cell cultures.

This study was conducted incorporating 2 independent tests. Ham's F-10 medium was the vehicle and cyclophosphamide and methylmethanesulfonate were the positive controls used in both tests. Xylanase was tested to the maximum permitted concentration of 5000 µg/ml in both tests.

The test, based on OECD Guidelines No. 473, ICH guidelines and with the European Commission Annex V, Test Method B10 (updated) was run at Inveresk Laboratories Tranent, Scotland during 19 April – 23 June 2004.

No toxicity was noted in the cultures harvested at 24 h. In the cultures harvested at 48 h (absence of the metabolizing enzyme mixture - S9 mix) toxicity was noted at concentrations of 1250 – 5000 µL. There was no evidence that Xylanase induced structural chromosomal aberrations in either the presence or absence of S9 mix. Xylanase did not induce polyploidy in the cultures harvested 48 h post treatment.

It was concluded that endo-1,4-β-xylanase was not clastogenic when tested with Chinese hamster ovary cells *in vitro*. *In vivo* tests were not performed, as there was no *in vitro* mutagenicity detected.

90-Day Sub-Chronic Toxicity Study

The test was performed according to the following guidelines: OECD No. 408 and toxicological principles for the safety assessment of direct food additives and color additives used in food, US FDA, 1982.

The systemic potential of endo-1,4-β-xylanase (test batch XT Mix Lims 2003-1463-1) was assessed in a 13-week oral study in rats. The test was designed and performed in accordance with the OECD

Guidelines No 408 adopted on 21.09.1998. The test was run at Inveresk Laboratories, Tranent Scotland during 28 June – 29 November 2004.

In this study, four groups of each 10 male and 10 female Sprague-Dawley rats were dosed daily for 13 weeks by gavage at levels of 0, 250, 500 or 1000 mg xylanase enzyme preparation/kg/day (raw enzyme preparation XT Mix with 94% TOS).

The animals were monitored daily for any signs of ill health or reaction to treatment. Detailed functional observations were performed weekly, with additional detailed functional observations performed during pre-treatment and during week 12 of treatment.
Body weights were recorded once during pre-treatment then daily throughout dosing. Food consumption was recorded once during pre-treatment and then weekly throughout treatment.

Water consumption was assessed visually on a weekly basis.

Ophthalmoscopic assessments were undertaken on all animals during pre-treatment and on all control and high dose animals during week 12.

Urine and blood samples were both collected for laboratory investigations during week 13. After 13 weeks of treatment, all surviving animals were killed and necropsied. All animals were given a detailed post mortem examination with major organs being weighed and/or placed in fixative. Tissues from all control and high dose animals and animals found dead were examined histologically. There were two premature decedents during the study. Histological examination of these animals indicated that their cause of death was not related to treatment with xylanase.

Daily oral dosing with endo-1,4-β-xylanase for 13 consecutive weeks was associated with a slight reduction in overall group mean body weight gain and an initial drop in food consumption performance in all treated female groups and in males treated at 500 or 1000 mg/kg/day. Softer than normal faeces were also noted throughout the study in all treated male groups and females dosed at

500 or 1000 mg/kg/day. Although these findings are regarded to be related to treatment with endo-1,4-β-xylanase, they are not considered to be of great toxicological significance.

There were no neurotoxic, other in-life, necropsy or histological findings that could be attributable to treatment with the test item.

In conclusion, a No Observed Adverse Effect Level (NOAEL) of 1000 mg endo-1,4-β-xylanase enzyme preparation/kg/day was derived, corresponding to an NOAEL of 940 mg TOS / kg bw / day.

Based upon the results of these studies, it can be concluded that the endo-1,4-β-xylanase enzyme preparation does not product adverse effects in rodents, nor was there any mutagenic or clastogenic activity detected.

C.2.3. Information on any Significant Similarity between the Amino Acid Sequence of the Enzyme and that of Known Protein Toxins.

Econase XT is the feed commercial name of the same enzyme preparation xylanase described herein (xylanase produced with the help of *Trichoderma reesei* RF5427) for its use in food. This enzyme was submitted to EU for its use in feed (see Appendix #9), part of the submission included a review of toxicity of the enzyme using bioinformatics. As such, we have provided in this dossier the study to analyze whether the xylanase has any significant amino acid sequences similarity with proteins identified as toxins.

A homology search was performed from the non-redundant protein sequences database using the BLAST-P (protein – protein BLAST) program, v. 2.2.30 (http://blast.ncbi.nlm.nih.gov/). The amino acid sequence of the Econase XT xylanase (Appendix #14 – treated as 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.

Control searches were conducted using five different shuffled versions of the Econase XT xylanase amino acid sequence as query sequences (Appendix 2). In these shuffled sequences the xylanase amino acid sequence is randomly rearranged but the overall amino acid composition of the sequence is retained. The shuffling of the xylanase amino acid sequence was performed using the Shuffle Protein Program of the Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/shuffle_protein.html). The range of E values obtained for the shuffled sequences represents the background control incidence of random hits that could be expected basing on the amino acids contained within the xylanase protein. The shuffled sequences were not expected to have significant homology to any other proteins in the database. In general the E value approaching zero indicates that there is a very low probability that such a match would occur randomly by chance.

According to the results obtained from the searches performed it can be concluded that the Rohalase® SEP Visco and Veron® HTX - xylanase (Econase XT) protein does not show significant homology to any protein sequence identified or known to be a toxin.

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

C.3.1. The source of the Enzyme Processing Aid

The enzyme is from a *Trichoderma reesei* host strain genetically modified with a xylanase gene deriving from *Thermopolyspora flexuosa* (previously named *Nonomuraea flexuosa*). The enzyme is not protein engineered.

Xylanase described in this application derives from *Nonomuraea flexuosa* DSM 43186 (ATCC 35864), which is an actinomycete known to produce thermostable xylanases (Holtz et al. 1991).

Actinomycetes are gram positive bacteria that can grow in filamentous form. They are common soil organisms.

Nonomuraea flexuosa DSM 43186 is **currently named as *Thermopolyspora flexuosa*** (Goodfellow et al. 2005). It was previously named as *Actinomadura flexuosa*, *Microtetraspora flexuosa or Nonomuria flexuosa (Zhang et al. 1998)*. As the name *Nonomuraea flexuosa* has been used in our publications on xylanases deriving from this strain, in this dossier both names *Thermopolyspora flexuosa* and *Nonomuraea flexuosa* are used for the donor organism. The taxonomic lineage of *Thermopolyspora* is shown below (according to http://www.uniprot.org/taxonomy/103836):

Genus:	Thermopolyspora
Species:	*Thermopolyspora flexuosa*
Subspecies (if appropriate):	not applicable
Generic name of the strain:	DSM 43186 (ATCC 35864)
Previous or other name(s) (if applicable):	*Nonomuraea flexuosa*
Commercial name:	Not applicable. The organism is not sold as such.

C.3.2. An Analysis of Similarity between the Amino Acid Sequence of the Enzyme and that of known Allergens.

As some enzymes manufactured for use in food have been reported to cause inhalation allergy in workers exposed to enzyme dust in manufacturing facilities, endo-1,4-β-xylanase may also cause such occupational allergy in sensitive individuals. However, the possibility of an allergic reaction to the endo-1,4-β-xylanase residues in food (mainly baked goods) seems remote. In order to address allergenicity by ingestion, it may be taken into account that:

- The allergenic potential of enzymes was studied by *Bindslev-Jensen et al. (2006)* and reported in the publication: "*Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry*". The investigation comprised enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and protein engineered variants and comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or

wasp. It was concluded from this study that ingestion of food enzymes in general is not likely to be a concern with regard to food allergy.

- Previously, the AMFEP Working Group on Consumer Allergy Risk from Enzyme Residues in Food performed an in-depth analysis of the allergenicity of enzyme products (Daurvin et al. 1998). The overall conclusion is that exposure to enzyme proteins by ingestion, as opposed to exposure by inhalation, are not potent allergens and that sensitization to ingested enzymes is rare.

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

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

- The majority of proteins are not food allergens and based on previous experience, the enzyme industry is not aware of any enzyme proteins used in food that are homologous to known food allergens.
- The food enzyme is used in small amounts during food processing, resulting in very small amounts of the enzyme protein in the final food. A high concentration generally equals a higher risk of sensitization, whereas a low level in the final food equals a lower risk (Goodman et al. 2008).
- In the case where proteins are denatured - which is the case for this endo-1,4-β-xylanase - due to the food process conditions (i.e baking process), the tertiary conformation of the enzyme molecule is destroyed. In general, these alterations in conformation are associated with decrease in the antigenic reactivity in humans: in the vast majority of investigated cases, denatured proteins are much less immunogenic than the corresponding native proteins (Valenta, Kraft 2002; Valenta 2002; Takai et al. 1997; Takai et al. 2000; Nakazawa et al. 2005; Kikuchi et al. 2006)
- In addition, residual enzyme still present in the final food will be subjected to digestion in the gastro-intestinal system, which reduces further the risk of enzyme allergenicity. While stability to digestion is considered as a potential risk factor of allergenicity, it is believed that small protein fragments resulting from digestion are less likely to be allergenic

- Finally, enzymes have a long history of safe use in food processing, with no indication of adverse effects or reactions. Moreover, a wide variety of enzyme classes (and structures) are naturally present in food. This is in contrast with most known food allergens, which are naturally present in a narrow range of foods.

In order to specifically evaluate the risk that the xylanase enzyme will cross react with known allergens and induce a reaction in an already sensitized individual, sequence homology testing to known allergens was performed. This test used a 80 amino acid (aa) sliding window search as well as conventional FASTA (overall homology), with the threshold of 35% homology and scanning with 6 mer for exact matches as recommended in the most recent literature (Food and Agriculture Organization of the United Nations January/2001; Ladics et al. 2007; Goodman et al. 2008).

The sequence homology comparison tests were performed using "AllergenOnline" database[3] , Allergen Database for Food Safety[4] , Structural Database of Allergen Proteins[5] and AllerMatch[6] . No indication of an allergenic potential of the xylanase was detected (Appendix #14 – treated as CCI).

Accordingly, it is concluded that the endo-1,4-β-xylanase preparation is not a potential allergen and no further allergenicity studies are necessary.

Conclusion

Xylanases of fungal and bacterial origin have been used in food for decades. We have no knowledge of any reports of allergic reactions to the residues of endo-1,4-β-xylanase in food as well as to the residues of other enzymes used in food processing.

By analogy, and on the basis of the results obtained from a sequence homology comparison test, and on the fact that the enzyme is typically denatured during the food manufacturing process and that any

[3] http://www.allergenonline.org
[4] http://allergen.nihs.go.jp/ADFS
[5] http://fermi.utmb.edu/SDAP/sdap_ver.html
[6] http://www.expasy.org/cgi-bin/lists?allergen.txt

residual enzyme still present in the final food will be subject to digestion in the gastro-intestinal system, it is not likely that the endo-1,4-β-xylanase produced by *Trichoderma reesei* under evaluation will cause allergic reactions after ingestion of food containing the residues of these enzymes.

Additional consideration; although some wheat derived product could be used as raw material during the fermentation process, AB Enzymes considers those materials derived from a major allergen used in the fermentation process to be consumed and absent in the final product (adequate carry over monitoring in place). Therefore, there are no known allergens in the enzyme preparation and thus no specific labeling requirement. This is in accordance with the EU Commission's position expressed to AMFEP (Association of Manufacturers and Formulators of Enzyme products).

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

France: The enzyme preparation was safety assessed according to the Guidelines for the evaluation of food enzymes[7] (EFSA GL, 2009). This resulted in the authorisation of the enzyme product by the French authorities. The approval letters from the French authorities and the ANSES scientific opinions are included in Appendix #7.

USA: A GRAS determination was notified to the US FDA in December 2015 (GRN000628, Appendix #8). In the reply letter from FDA dated September 14th, 2016, the agency has no questions regarding AB Enzymes' determination that the xylanase enzyme reparation is GRAS for its intended use.

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

D.1. Information on the source organism

The microorganism that is used for the production of endo-1,4, β-xylanase, is the fungus *Trichoderma reesei*.

[7] http://onlinelibrary.wiley.com/doi/10.2903/j.efsa.2009.1305/epdf

Scientific name:

Genus:Trichoderma

Species: *Trichoderma reesei*

Taxonomy: *Trichoderma reesei* is a hypercellulolytic fungus which was found on deteriorating military fabrics such as tents and clothing. This isolate, designated as QM6a, was initially named *Trichoderma viride*. Approximately 20 years later, QM6a was re-classified as *Trichoderma reesei*. In the 1980s, it was suggested that *Trichoderma reesei* should be placed into synonymy with *Trichoderma longibrachiatum* (Bissett 1991). Later however, evidence appeared that the two species were not identical (Meyer et al. 1992) and it was decided to go back to the *Trichoderma reesei* name. It is of relevance to note that enzymes have been approved that are produced by *T. reesei* under the name of *T. longibrachiatum*[8]. Taxonomic studies have shown that the species *Trichoderma reesei* consists only of this single isolate QM6a and its derivatives (e.g. Rut Series, Montenecourt and Eveleigh, 1977, 1979; QM9123 and QM9414, Mandels et al, 1971 – as reviewed by Nevalainen et al. (1994)). The American Type Culture Collection (ATCC) designation for this original strain of *Trichoderma reesei* QM6a is ATCC 13631.

Synonyms[9]**:** T*richoderma reesei* is the species name given to the anamorphic form (the form which reproduces asexually) of the fungus whose telemorphic form (the form which reproduces sexually) is now understood to be *Hypocrea jecorina* (Kuhls et al. 1996; Seidl et al. 2008). *Trichoderma reesei* was formerly known as *Trichoderma longibrachiatum*.

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

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

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

Islands in 1944. As *T. reesei is a goo*d producer of cellulases, it has been widely studied in several laboratories and developed as industrial enzyme producer using random mutagenesis and genetic engineering. The original isolate, QM6a is the initial parent of practically all currently industrially relevant food enzyme production strains, including our strain RF5457.

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

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

Pathogenicity:

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

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

- In the USA, *Trichoderma reesei* is not listed as a Class 2 or higher Containment Agent under the National Institute of Health Guidelines for Recombinant DNA Molecules. Data submitted in Generally Recognized as Safe (GRAS) petitions to the Food and Drug Administration (FDA) for numerous enzyme preparations from T. *reesei* for human and animal consumption demonstrate that the enzymes are nontoxic. The Environmental Protection Institute (EPA) completed a risk assessment on *T. reesei* in 2011 resulting in a Proposed Rule in 2012, concluding that it is appropriate to consider *T. reesei* as a recipient microorganism eligible for exemptions from full reporting requirements[10], if

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

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

- In Europe, *Trichoderma reesei* is classified as a low-risk-class microorganism, as exemplified by being listed as Risk Group 1 in the microorganism classification lists of the German Federal Institute for Occupational Safety and Health (BAuA[11]) and the Federal Office of Consumer Protection and Food Safety (BVL), and not appearing on the list of pathogens from Belgium (Belgian Biosafety Server, 2010[12]).

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

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

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

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

— Under controlled industrial fermentation conditions, the organisms are not subjected to significant stress: the literature indicates that the biosynthesis of peptaibols is a defence response against other fungi when subjected to environmental stress such as the lack of nutrients (Tisch, Schmoll 2010; Komon-Zelazowska et al. 2007).

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

[12] http://www.biosafety.be/RA/Class/ClassBEL.html

— Standard industrial fermentation process times are short for peptaibols induction: peptaibols have mostly been isolated from very old cultures of *Trichoderma*, at least 15 days of cultivation (Kubicek et al. 2007). Industrial fermentation processes for *Trichoderma reesei* can be up to 10 days, but is typically shorter (3-8 days).

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

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

T. reesei RF5427 fermentation extracts have been subjected to several tests as part of their safety assessment for the production of food products. In toxicological tests that have been performed, including a 90-day repeated dose study, no toxicity of xylanase fermentation product as produced by the present production strain *Trichoderma* reesei RF5427 was detected (see **Section C**). These results show that there is no need for any toxicological concern with fermentation products as produced by use of *Trichoderma reesei*.

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

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

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

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

This section contains summarized information. The detailed information is provided in the Appendix #14 – treated as CCI.

Host organism

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

The *Trichoderma reesei* host strain is genetically modified with a xylanase gene deriving from *Thermopolyspora flexuosa* (previously named *Nonomuraea flexuosa*).

Donor

The endo-1,4-ß-xylanase described in this dossier was derived from *Thermopolyspora flexuosa* (*Nonomuraea flexuosa*) DSM43186 which is an actinomycete known to produce thermo-tolerant xylanases. Actinomycetes are gram positive bacteria that can grow in filamentous form. They are common soil organisms.

Genetic modification

Trichoderma reesei strain RF5427 was constructed for production of *Thermopolyspora flexuosa* (*Nonomuraea flexuosa)* derived xylanase by introducing the encoding gene into the genome of the *Trichoderma reesei* host.

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

It consists of a *T. reesei* signal sequence and a carrier polypeptide encoding sequences, the *Thermopolyspora flexuosa* (*Nonomuraea flexuosa)* derived xylanase coding sequence and *Aspergillus nidulans amdS* gene sequence (as selection marker).

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

Stability of the transformed genetic sequence

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

The stability and potential for transfer of genetic material was assessed as a component of the safety evaluation of the production microorganism. Southern blot analyses were performed to the genome of the *T. reesei* production strain RF5427. Results indicated that several copies of the expression cassettes were integrated in the genome of strain RF542 and that the production strain is stable in terms of genetic traits.

For more details, please see Appendix #14 – treated as CCI.

F. Information Related to the Dietary Exposure to the Processing Aid

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

The food enzyme object of the dossier is typically used in baking process and processes of other cereal based products (such as pasta and noodles), grain processing, distilling, and brewing.

In principle, the enzymatic conversion of (arabino)xylans with the help of endo-1,4-β-xylanase can be used in the processing of all food raw materials which naturally contain(arabino)xylans.

Commercial food enzyme preparations are generally used following the *Quantum Satis* (QS) principle, i.e. at a level not higher than the necessary dosage to achieve the desired enzymatic reaction – according to Good Manufacturing Practice. The amount of enzyme activity added to the raw material by the individual food manufacturer has to be determined case by case, based on the desired effect and process conditions. Therefore, the enzyme manufacturer can only issue a recommended enzyme dosage range. Such a dosage range is the starting point for the individual food producer to fine-tune his process and determine the amount of enzyme that will provide the desired effect and nothing more. Consequently, from a technological point of view, there are no 'normal or maximal use levels' and endo-1,4-β-xylanase is used according to the QS principle. A food producer who would add much higher doses than the needed ones would experience untenable costs as well as negative technological consequences.

Microbial food enzymes contain – apart from the enzyme protein in question – also some substances derived from the producing micro-organism and the fermentation medium. The presence of all organic materials is expressed as Total Organic Solids[13] (TOS, FAO/WHO, 2006). Whereas the dosage of a food enzyme depends on the enzyme activity present in the final food enzyme preparation, the dosage on basis of TOS is more relevant from a safety point of view. Therefore, the use levels are expressed in TOS. The Table below shows the range of applications where the endo-1,4-β-xylanase is intended to be used.

Foods Uses for Xylanase	
Food Grouping	**Proposed Food Uses**
Cereals and cereal products	Flours and other cereal grains and starches Regular breads and rolls Fancy breads, etc.

[13] 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.

	Pasta and pasta products
Cereal-based products and dishes	Sweet pastries Savoury biscuits Cakes, buns, muffins etc.
Alcoholic beverages	Spirits, Beer
Miscellaneous	Starch, fibers, gluten used in – soft drinks and beverages (see description in Section F.2 for details regarding this food application)

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	Total solid food	Total non-milk	Processed food	Soft drinks

lifetime/kg body weight/day	(kg)	beverages (l)	(50% of total solid food) (kg)	(25% of total beverages) (l)
	0.025	*0.1*	*0.0125*	*0.025*

The recommended use levels of endo-1,4-β-xylanase are given based on the raw materials used in the food processes. For the calculation of the TMDI, the maximum use levels are chosen. Furthermore, the calculation takes into account how much food (or beverage) is obtained per kg raw material and it is assumed that all the TOS will end up in the final product and the wide variety of food products based on edible oils that are available to consumers. In the case of alcohol distillation, however, it is assumed that nothing of the TOS will end up in the final product due to the distillation process. Therefore, this application is not mentioned in the Table below.

Applications		Raw material (RM)	Recommended use level (mg TOS/kg RM)	Final food (FF)	Ratio RM/FF*	Maximal level in final food (mg TOS/kg food)
Liquid foods	Alcoholic beverages	Cereals	5	Beer	0.17	0.85
	Miscellaneous	Cereals	10	Starch, fibers, gluten used in – soft drinks and beverages	1.1	**11**

Solid foods	Baking and other cereal products	Flour	10	Bread, baked goods, etc.	0.71	10
	Grain processing	Cereals	10	Starch, fibers, gluten used in – soft drinks and beverages	1.1	**11**

* Assumptions behind ratios of raw material to final food*:*

Baking

- *Bakery products fall in the category of solid foods.*
- *Flour is the raw material for bakery product and the yield will vary depending on the type of final food produced. From 1 kg of flour you would have 4 kg of cakes, 1.4 kg of bread or 1.1 kg of cracker. Cracker may represent the most conservative input from the bakery processes. However, consumption of bread is higher than that of cracker, 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.*

Brewing and cereal drinks

- *Brewing and cereal drinks add to the class of liquid foods.*
- *Raw materials used in brewing and cereal drink processes are various kinds of grist (e.g. malt, barley, wheat, sorghum and maize). Yields will vary dependent on the type of grist, process used and the type of drink produced.*
- *Beer production has a range of RM/FF from 14-28 kg of grist per 100 L of beer, with 80-90 % of all beers produced at a RM/FF ratio of 14-20 kg of grist per 100 L of beer. The same RM/FF ratio holds true for cereal beverage.*
- *The assumption used for calculation of dietary exposure is a yield of 100 L of drink per 17 kg of cereal corresponding to a RM/FF ratio of 0.17 kg grist per L of beer or cereal beverage.*

Grain processing

Food ingredients obtained from grain processing are typically Starch, Fibre, Gluten and Flour. These food ingredients can be use in the making of both solid and liquid final foods.

Grain processes might start with cereals (grains or grist) or flour as the raw material. Cereals contain starch in a range of 55-65%, fibre in the range of 6-18%1 and gluten in the range of 10-15%.

- *Starch: Typically 0.55 kg starch is produced per 1 kg cereal. The most considerable final food application is dairy and bakery with a maximum added starch content of 5%. Starch is also used in the less voluminous application area of confectionary, where it is used up to a content of 12%. Based upon the most considerable applications (bakery), the corresponding RM/FF ratio is 0.09 kg cereal per kg final food (same for dairy).*

  Starch can also be further processed into syrups (e.g. High Fructose Corn Syrup, HFCS), sweeteners and modified starch (Starch processing). Syrups and sweeteners are mainly used in liquid foods (soft drinks). With the assumptions expressed above (typically 0.55 kg starch is produced per 1 kg cereal) and assuming that typically 1 kg of sweetener is produced per 1 kg starch, and that soft drinks typically contain 10-14% w/v HFCS so on average 120 g HFCS per L, it can be concluded that the typical ratio of RM/FF is 0.21 kg cereals per L final beverage.
- *Fibre: Typically 0.12 kg fibre is produced per 1 kg cereal. Fibre is used in bakery and beverage products with a maximum added fibre content of 13% (total fibre content max. 25%). The corresponding RM/FF ratio is 1.1 kg cereal per kg final food.*
- *Gluten: Typically 0.10 kg gluten is produced per 1 kg cereal. Gluten is used in the production of bakery products with a maximum added gluten content of 10% in the final food. The corresponding RM/FF ratio is 1 kg cereal/kg final food.*

In respect to dietary exposure calculation, the worst case scenario, both in respect to solid and liquid food, is food ingredient Fibre/cereals with a RM/FF ratio of **1.1 kg cereal per kg final food.**

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

The Total TMDI will consequently be: TMDI in food (mg TOS/kg body weight/day)	TMDI in beverage (mg TOS/kg body weight/day)	Total TMDI (mg TOS/kg body weight/day)
11 x 0.0125 = 0.1375	11 x 0.025 = 0.275	**0.4125**

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

- It is assumed that ALL producers of the above mentioned foodstuffs (and beverages) use the specific enzyme endo-1,4-β-xylanase from *Trichoderma reesei*;
- It is assumed that ALL producers apply the HIGHEST use level per application; For the calculation of the TMDI's in food, only THOSE foodstuffs were selected containing the highest theoretical amount of TOS. Thus, foodstuffs containing lower theoretical amounts were not taken into account;
- It is assumed that the amount of TOS does not decrease as a result of the food production process;
- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed DAILY over the course of a lifetime;
- Assumptions regarding food and beverage intake of the general population are overestimates of the actual average levels *(Douglass et al. 1997)*.

The Margin of Safety (MoS) for human consumption can be calculated by dividing the NOAEL by the Total Theoretical Maximal Daily Intake (TMDI). As was shown in **Section C.2.2**, a No Observed Adverse Effect Level (NOAEL) of 1000 mg endo-1,4-β-xylanase enzyme preparation/kg/day was derived, corresponding to an NOAEL of 940 mg TOS / kg bw / day.

Consequently, the MoS is: MoS = 940 / 0.4125 = **2,279**

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

The overall conclusion is that the use of the food enzyme endo-1,4-b-xylanase from *Trichoderma reesei* RF5427 in the production of food is absolutely safe. Considering the high safety factor – even when calculated by means of an overestimation of the intake via the Budget method – there is no need to restrict the use of the enzyme in food processing.

Consequently, it is concluded that enzyme endo-1,4-b-xylanase from *Trichoderma reesei* RF5427 can be used *Quantum Satis* in baking, brewing, grain processing and production of potable alcohol.

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 xylanase enzyme as a processing aid at the recommended dose.

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

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

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

Not applicable.

VI. List of appendices

Section 3.1

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Section 3.3

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