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APPLICATION

SEPTEMBER 2018

TO:
FOOD STANDARDS AUSTRALIA NEW ZEALAND (FSANZ)

IN RELATION TO:
APPLICATION FOR APPROVAL OF ENDO-INULINASE FROM A GENETICALLY MODIFIED STRAIN OF *ASPERGILLUS ORYZAE* AS A PROCESSING AID

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CONFIDENTIAL COMMERCIAL INFORMATION (CCI) INFORMATION

Additional material has been provided under CCI to support the Application for Sections 6.1.3; 6.1.4, 6.4.1 and 6.4.2. This material has been provided separately to this Application document.

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ADMINISTATIVE INFORMATION

(As per section 3.1.1 B of the Application Handbook as at 1 March 2016)

Applicant:

Organization: Puratos NV (Puratos)

Address: Industrialaan 25, B-1702 Groot-Bijgaarden, Belgium

Telephone:

Email address:

Primary contact: Fiona Fleming

Nature of Business

(As per section 3.1.1 B of the Application Handbook as at 1 March 2016)

Puratos is a company specialising in the development, production, distribution and marketing of high-quality raw materials for the bakery, confectionery, chocolate and catering industry.

Details of other parties associated with the Application

(As per section 3.1.1 B of the Application Handbook as at 1 March 2016)

1. The following Scientific and Regulatory Consultants have been involved in the preparation, submission and stewardship of this application:

    - Dr Simon Brooke-Taylor, Brooke-Taylor & Co Pty Ltd
    - Fiona Fleming, FJ Fleming Food Consulting Pty Ltd

2. The following manufacturer has an interest in the application:

    Company: Beldem – a division of Puratos NV

    Address: rue Bourrie 12, B-5300 Andenne, Belgium

    Telephone:

1. APPLICATION INFORMATION

Status of Similar Applications

(As per Section 3.1.1 D of the Application Handbook as at 1 March 2016)

There are no similar current applications for approval of this specific endo-inulinase (EC 3.2.1.7) cloned in *Aspergillus oryzae* MUCL 44346 as a processing aid.

Assessment Procedure

(As per section 3.1.1 F of the Application Handbook as at 1 March 2016)

Puratos seeks to submit this application for consideration as a General Procedure (maximum of 350 hours).

Puratos will confirm whether the application will be paid or unpaid following completion of the Administrative Assessment by FSANZ.

Confidential commercial information (CCI)

(As per section 3.1.1 G of the Application Handbook as at 1 March 2016)

This application **does contain** information that is confidential commercial information (CCI).

Puratos has provided information to support this application which it considers to be CCI. This information is provided separately and clearly labelled as CCI.

Exclusive capturable commercial benefit [ECCB]

(As per section 3.1.1 I of the Application Handbook as at 1 March 2016)

This application will **not confer** an exclusive capturable commercial benefit for Puratos or any other individual company.

2. PURPOSE OF THE APPLICATION

(As per section 3.1.1 D of the Application Handbook as at 1 March 2016)

Puratos is making this application to amend Schedule 18 – Processing Aids, of the Australia New Zealand Food Standards Code (hereafter the Code) to include the food enzyme endo-inulinase (EC 3.2.1.7) cloned in *Aspergillus oryzae* MUCL 44346 in S18-9(3) Permitted Enzymes.

The food enzyme shows inulinase activity as defined under IUBMB No EC 3.2.1.7 and is used as a processing aid in the production of fructo-oligosaccharides (FOS) as shown in **Figure 1.** FOS is a non-standardised food in the Code and exempted from the definition of "used as a nutritive substance" under 1.1.2-12 – Definitions used throughout the Code.

The purpose is for approval of the enzyme as a processing aid as it is not currently approved for use in Australia and New Zealand.

3. JUSTIFICATION FOR THE APPLICATION

(As per section 3.1.1 D of the Application Handbook as at 1 March 2016)

3.1 NEED FOR THE PROPOSED CHANGE

(As per section 3.1.1 D of the Application Handbook as at 1 March 2016)

Schedule 18 - Processing Aids contains a list of permitted enzymes of microbial origin (S18-4 Permitted Enzymes). There is already approval for Inulinase (EC 3.2.1.7) from *Aspergillus niger.*

The source microorganism for the applicant's endo-inulinase is *Aspergillus oryzae.*

Approval is required due to the use of a new source microorganism for the preparation of the enzyme.

This application will provide information to support the safety of the endo-inulinase enzyme and the genetically modified *Aspergillus oryzae* as a host organism.

3.1.1 Purpose of using the processing aid

Like any other enzyme, endo-inulinase acts as a biocatalyst - with the help of the enzyme, a certain substrate is converted into a certain reaction product.

The **function** of endo-inulinase is to catalyse the Endohydrolysis of (2→1)-β-D-fructosidic linkages in inulin.

In general, the technological need of the enzymatic conversion of inulin with the help of endo-inulinase can be described as the production of fructo-oligosaccharides (FOS) as a sugar alternative for sucrose.

The **substrate** for endo-inulinase is inulin. Inulin is polysaccharides composed of fructose unit chains of various length terminated by a single glucose unit which can be found in Jerusalem artichoke and chicory (*Zittan 1981*). Consequently, the substrate for endo-inulinase occurs naturally in foods.

The **reaction product** of the hydrolysis of inulin with the help of endo-inulinase is a syrup of fructo-oligosaccharides (FOS).

Endo-inulinase performs its technological function during FOS production. The enzyme does not perform any technological function in the final food.

3.2 Advantages of the Proposed Change

(As per section 3.1.1 D of the Application Handbook as at 1 March 2016)

The beneficial effects mentioned in the application below are of value to the food chain because they lead to the approval of an enzyme that can be used for the production of fructo-oligosaccharides (FOS) as sugar alternative.

The different inulinases have different pH & temperature optima, giving additional options for FOS producers. Approval of this enzyme offers an alternative to other enzymes available, so manufacturers of FOS have a choice and are not tied to one supplier or process.

3.3 Disadvantages of the Proposed Change

(As per section 3.1.1 D of the Application Handbook as at 1 March 2016)

The Applicant is not aware of any disadvantages of the proposed change.

3.4 Public Health and Safety Issues

(As per section 3.1.1 D of the Application Handbook as at 1 March 2016)

The Applicant has not identified any public health and safety issues in relation to the approval of endo-inulinase from *Aspergillus oryzae* for use in the Australia/New Zealand food supply.

Refer **Section 6** for information about the safety of the processing aid.

3.5 Consumer Choice

(As per section 3.1.1 D of the Application Handbook as at 1 March 2016)

No consumer choice issues related to the proposed change are foreseen.

Endo-inulinase does not perform any technological function in the final foods containing ingredients prepared with this enzyme. Moreover, the food products prepared with this enzyme do not have characteristics or nutritional value other than what is expected by the consumer.

3.6 SUPPORT FOR THE PROPOSED CHANGE

(As per section 3.1.1 D of the Application Handbook as at 1 March 2016)

The Applicant does not have letters from potential customers, however the Australia/New Zealand business (Puratos Australia-New Zealand Pty Ltd) intends to market the enzyme in Australia once it is approved.

3.7 REGULATORY IMPACT INFORMATION

(As per section 3.1.1 D.1 of the Application Handbook as at 1 March 2016)

3.7.1 Costs and Benefits of the application

(As per section 3.1.1 D.1.1 of the Application Handbook as at 1 March 2016)

Costs and Benefits – Consumer

The potential benefit to consumers includes:

- choice of additional products which become available due to the availability of endo-inulinase for Australian and New Zealand food manufactures, and
- access to food products that are currently manufactured overseas with the use of endo-inulinase.

The proposed amendment places no additional economic cost on consumers.

Costs and Benefits - Industry and Business

The decision to use this enzyme is a technical one taken by the manufacture based on the FOS profile they wish to achieve.

The benefits for industry that may be obtained in the final foods when produced with the help of endo-inulinase may include:

- additional enzymatic option for the creation of FOS
- creation of FOS which are highly soluble and have technological properties similar to those of sugar and glucose, and
- creation of FOS which can be used as a low caloric bulking agent to replace sugar.

Use of endo-inulinase will be at the discretion of business, therefore there are no direct costs imposed on industry.

Costs and Benefits – Government

The proposed amendment places no additional regulatory costs on government beyond the initial regulatory cost of approving this endo-inulinase enzyme as a processing aid.

3.7.2 Impact on International Trade

(As per section 3.1.1 D.1.2 of the Application Handbook as at 1 March 2016)

The Applicant notes that, in developing food standards, FSANZ must have regard to its WTO obligations; the promotion of consistency between domestic and international food standards; and the promotion of fair trading in food. These matters encompass consideration of international standards and trade issues.

This amendment would bring Australia and New Zealand into line with other countries where endo-inulinase is permitted for use (outlined under Section 5).

4 INFORMATION TO SUPPORT THE APPLICATION

(As per section 3.1.1 E of the Application Handbook as at 1 March 2016)

4.1 Data Requirements

The data and information provided to support the application fulfils the requirements for data as set out in the FSANZ Application Handbook:

- the source, author and year of evidence is identified
- it has been obtained using validated methods
- it represents Australian and New Zealand populations where possible, and
- it has been compiled from studies conducted under good laboratory practice (GLP).

Refer to **Section 6** for information about the processing aid.

4.2 FSANZ Act Objectives

Information is provided in this application to address the objectives specified in Section 18 of the FSANZ Act as follows:

(a) The protection of public health and safety - information to support objective (a) is provided in the following sections of the Application:

- 6.2 - Information on the Safety of an Enzyme Processing Aid.
- 6.3 - Additional Information Related to the Safety of an Enzyme Processing Aid Derived from a Microorganism.
- 6.4 - Additional Information Related to the Safety of an Enzyme Derived from a Genetically-Modified microorganism.
- 6.5 – Information related to the Dietary Exposure to the processing aid.

(b) The provision of adequate information relating to food to enable consumers to make informed choices.

Processing aids are not required to be labelled however consumers are able to contact manufactures to request information in relation to finished products if they have an interest or query.

(c) The prevention of misleading or deceptive conduct.

There are no reasons to assume that the use of this food enzyme could lead to a food which would be misleading in terms of nature, freshness, the quality of the ingredients used or the nutritional quality of the final food. The application of food enzymes does not lead to the potential use of raw materials of inferior or unsafe quality. Instead, food enzymes are used to compensate natural variations of the agricultural raw material; liberate the full potential of the raw material and to support production processes which are more environmentally friendly. Enzymatic processes in general occur in nature and they therefore do not influence the “naturalness” of the production process or the final food. Considering the above, there are no reasons to believe that the use of endo-inulinase could be misleading for the consumer.

4.3 POLICY GUIDELINES

(As per section 3.3.2 of the Application Handbook as at 1 March 2016)

Information is provided in this application to address the Policy Guideline - Addition to Food of Substances other than Vitamins and Minerals.

Addition to Food of Substances other than Vitamins and Minerals

The addition of substances other than vitamins and minerals to food where the purpose of the addition is to achieve a solely technological function should be permitted where:

Specific Order Policy Principles – Technological Function	Section of Application
a) the purpose for adding the substance can be articulated clearly by the manufacturer (i.e. the ‘stated purpose’); and	2 & 3.1
b) the addition of the substance to food is safe for human consumption; and	6
c) the amounts added are consistent with achieving the technological function; and	6
d) the substance is added in a quantity and a form which is consistent with delivering the stated purpose; and	6
e) no nutrition, health or related claims are to be made in regard to the substance.	Not applicable

5 INTERNATIONAL AND OTHER NATIONAL STANDARDS

(As per section 3.1.1 J of the Application Handbook as at 1 March 2016)

The status of the processing aid with respect to other national standards or regulations is discussed under this section of the Application.

5.1 INTERNATIONAL STANDARDS

(As per section 3.1.1 J.1 of the Application Handbook as at 1 March 2016)

Endo-inulinase is listed in the IPA database by CCFA (refer to **Appendix 1**).

5.2 OTHER NATIONAL STANDARDS OR REGULATIONS

(As per section 3.1.1 J.2 of the Application Handbook as at 1 March 2016)

5.2.1 Existing Authorisations and Evaluations

The food enzyme endo-inulinase has been evaluated and/or authorized in the USA as set out in Table 1. Moreover, the food enzyme endo-inulinase has been submitted for evaluation to the JECFA and has been legally produced and used in the EU, where it has also been submitted for evaluation to the EFSA. The date of submission was 30/11/2015 – the application is currently in progress with an unknown completion date.

Table 1: Non-exhaustive list of authorisations of endo-inulinase produced by *Aspergillus oryzae*

Authority	Description	Reference
USA[1]	Self-affirmed GRAS[2]	Appendix 2

5.2.2 Other Authorisations and Evaluations

Similar food enzymes have not as yet been evaluated by EFSA. However, within the EU and internationally, food enzymes similar to the one described in this application have already been evaluated.

Food enzymes are biological isolates of variable composition. Apart from the enzyme protein in question, microbial food enzymes will also contain some substances derived from the producing micro-organism and the fermentation medium. From a safety point of view, the similarity of the producing micro-organism is of higher importance than that of the enzyme protein in question.

Therefore, the non-exhaustive lists below summarize not only authorized food enzymes with the same enzyme activity, but also authorized food enzymes from the same producing organism. Authorised food enzymes other than endo-inulinase from the same production organism

1 GRAS affirmations and GRAS notifications

2 There is no GRAS number as this is Self-Affirmed

(notwithstanding any genetic modifications) (Table 2), and authorised endo-inulinase from production organisms other than *A. oryzae* (Table 3).

Table 2: Non-exhaustive list of authorized food enzymes (other than endo-inulinase) produced by the same production organism, *Aspergillus oryzae*

Authority	Food enzyme	Reference
JECFA	Alpha-amylase	Appendix 3
	carbohydrase	
	lipase	
	protease	
	laccase	
Australia / New Zealand	Aminopeptidase	ANZ Food Standards Code Schedule 18 – Permitted Enzymes – 18-4 (Copy not provided with Application)
	α–Amylase	
	Asparaginase	
	β-Galactosidase	
	β-Glucanase	
	Glucoamylase	
	Glucose oxidase	
	α-Glucosidase	
	Lipase, triacylglycerol	
	Metalloproteinase	
	Mucorpepsin	
	Pectinesterase	
	Phospholipase A_1	
	4-Phytase	
	Polygalacturonase	
	Serine proteinase	
Canada	Amylase	Appendix 4
	Asparaginase	
	Glucoamylase	
	Glucose oxidase	
	Lactase	
	Lipase	
	Milk coagulating enzyme	
	Pectinase	
	Phospholipase	
	Protease	
	Xylanase	
France	Alpha amylase	Arrêté du 19 Octobre 2006
	Aminopeptidase	

Table 2: Non-exhaustive list of authorized food enzymes (other than endo-inulinase) produced by the same production organism, *Aspergillus oryzae*

Authority	Food enzyme	Reference
	Amyloglucosidase	Appendix 5
	Asparaginase	
	Aspartyl protéase	
	Bêta galactosidases	
	Glucose oxydase	
	Lactases	
	Lipase	
	Pectine méthylestérase	
	Phospholipase A1	
	Protéase	
	Tannase	
	xylanase	
USA	Asparaginase	Appendix 2
	Phospholipase	
	Laccase	
	Lipase	
	Glucose oxidase	
	Carbohydrase	
	Aspartic proteinase	
	Exopeptidase	
	Pectin esterase	

Table 3: Non-exhaustive list of authorized endo-inulinase from production organisms other than *Aspergillus oryzae*		
Authority	**Production organism**	**Reference**
Australia / New Zealand	*Aspergillus niger*	ANZ Food Standards Code - Schedule 18 – Permitted Enzymes – 18-4 (Copy not provided with Application)
Canada	*Aspergillus niger*	Appendix 4
France	*Aspergillus niger*	Appendix 5

6 SUBSTANCES ADDED TO FOOD - PROCESSING AIDS

(As per section 3.3.2 of the Application Handbook as at 1 March 2016)

6.1 TECHNICAL INFORMATION ON THE PROCESSING AID

(As per section 3.3.2 A of the Application Handbook as at 1 March 2016)

The material described in this section is representative of the commercial product.

6.1.1 Information on the type of processing aid

(As per section 3.3.2 A.1 of the Application Handbook as at 1 March 2016)

The processing aid which is the subject of this application is a specific endo-inulinase (EC 3.2.1.7) cloned in *Aspergillus oryzae* MUCL 44346.

The application request is for approval of this enzyme as a permitted processing aid – various technological purposes (S18-9). The food enzyme shows inulinase activity as defined under IUBMB No EC 3.2.1.7 and is used as a processing aid in the production fructo-oligosaccharides (FOS).

Information on the processing aid & Evidence that the form and the amount of the processing aid performs the intended function

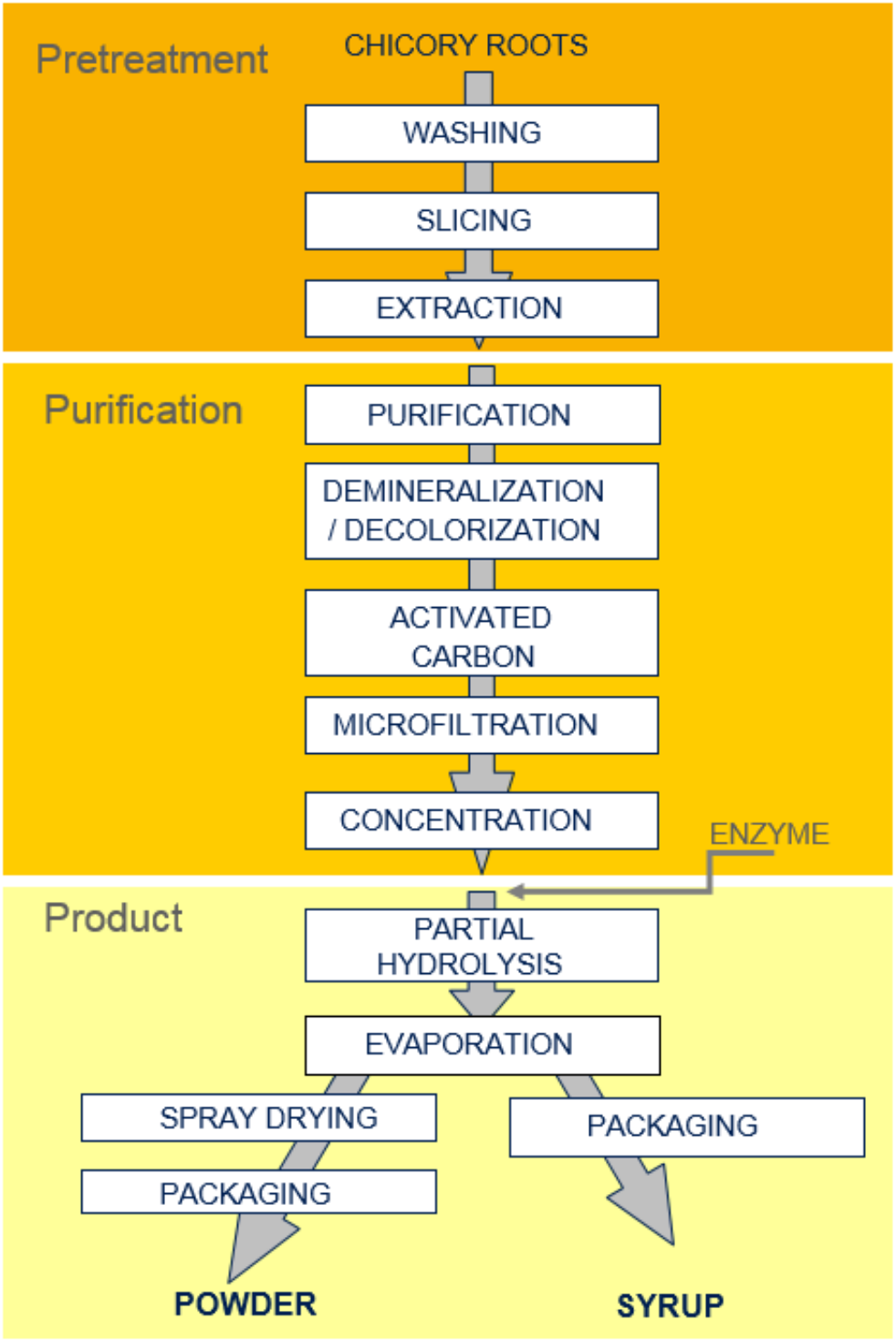
The enzymatic conversion of inulin with the help of endo-inulinase is a specific process as shown in **Figure 1** below.

The individual inulin processors consider pH and temperature stability of the enzyme as relevant for having the technological functionality of the food enzyme during processing.

Generally, the pH range for the hydrolysis ranges from pH 5 to pH 6 and the temperature ranges from 54 to 56^{0}C is based on enzyme activity and other optimizations.

The hydrolysis is followed by concentration to approximately 75% dry solids by evaporation for approximately 10 minutes at temperatures starting at 95°C and ending at slightly below 80°C. Those temperature conditions inactivate the enzyme in the final product. Three recent batches have been tested on rest activity in the final product (**Appendix 6**). As can be seen, no significant rest activity can be measured, confirming the inactivation of the enzyme during these final steps.

Figure 1: Production of chicory fructo-oligosaccharies (FOS)/oligofructose



6.1.2 Information on the identity of the processing aid

(As per section 3.3.2 A.2 of the Application Handbook as at 1 March 2016)

Enzyme

Name of the enzyme protein:	inulinase (accepted IUBMB name)
Synonyms:	inulase; indoinulinase; endo-inulinase; exoinulinase; 2,1-β-D-fructan fructanohydrolase
Abbreviations:	None
EC (IUBMB) number:	EC 3.2.1.7
Protein Engineered	The enzyme has NOT been protein engineered

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

EC 3 Hydrolase
EC 3.2 Glycosylase
EC 3.2.1 Glycosidases
EC 3.2.1.7 Inulinase

Enzyme Preparation

Commercial Name:

This enzyme is already sold under different commercial names:

- Oligofruct'Ase

A Technical Data Sheet is provided as **Appendix 7.**

Host Organism

Name:

The organism is deposited under the number MUCL- 44346 at the node of the Belgian Coordinated Collection of Microorganisms located at the Louvain-la-Neuve.

The address of the Culture Collection is:

Mycothèque de l'Université catholique de Louvain, Croix du Sud 2, box L7.05.06, B-1348 Louvain-la-Neuve, Belgium

Donor Organism

The microorganism used as donor strain, is *Aspergillus ficuum ATCC16882*. According to the current state of the art, the taxonomic classification of this microorganism is as follows:

Genus:	*Aspergillus*
Species:	*Aspergillus ficuum*
Synonyms[3]:	*Ustilago ficuum; Sterigmatocystis ficuum; Aspergillus niger var. ficuum; Aspergillus batatas; Aspergillus batatae; Sterigmatocystis batatae*

The description of the genetic modification is described in the CCI section 6.4.1.3

6.1.3 Information on the chemical and physical properties of the processing aid

(As per section 3.3.2 A.3 of the Application Handbook as at 1 March 2016)

Commercial enzymes, whether used in the production of food, feed or for technological purposes, are biological isolates of variable composition. Food enzymes are concentrates containing a specific enzyme protein, whose activity (also called main or principal activity) can be used for a specific, intended technological purpose in food processing. Apart from the enzyme protein in question, microbial food enzymes also contain some substances derived from the producing microorganism and the fermentation medium. These constituents consist of organic material (proteins, peptides, amino acids, carbohydrates, lipids) and inorganic salts. As has been established by JECFA (FAO/WHO, 2006), the percentages of these organic materials are summarized and expressed as Total Organic Solids (TOS). The TOS value is an internationally accepted method to describe the chemical composition of commercial food enzymes. The ratio between the enzyme activity and TOS is an indication of the relative purity of the enzyme.

Protein content and relative purity of the food enzyme endo-inulinase from *Aspergillus oryzae* was measured and the TOS values were calculated in 3 batches. The result is shown in the following Table.

3 Reference: Mycobank taxonomic database (see: http://www.mycobank.org/Biolomics.aspx?Table=Mycobank&Page=200&ViewMode=Basic). More synonyms can be found under each of the names mentioned.

Table 4: Composition and Specification of the Commercial food enzyme

Batch No	INU1401	INU1402	INU1407	Mean
Ash (%)	0.08	0.1	0.18	0.12
Water (%)	98.23	97.28	97.92	97.81
TOS (%)	1.69	2.63	1.9	2.07
Activity (IU/ml)	2461	3032	3548	3013
Units/mg TOS	145.62	115.28	186.74	145.55
Protein (g/L)	10.75	12.45	4.19	9.13

Certificates of Analysis (COA) are provided in **Appendix 8.**

The methods by which the ash and dry matter content (to calculate the TOS) and protein values are measured are standardized and/or validated methods and given in **Appendix 8.**

The method, by which the enzyme activity is measured, including an explanation of the units, is given in **Appendix CCI A1.**

With respect to the potential for specific chemicals which are used in the fermentation and downstream processing to end up in the food enzyme - please refer to Section 6.1.4 - manufacturing process.

Information on the specific properties of the enzyme protein, such as Molecular Mass, amino acid sequence, post translational modification are provided in Section 6.1.3.1.

It is proposed that the food enzyme endo-inulinase should comply with the internationally accepted JECFA specifications for chemical and microbiological purity of food enzymes (FAO/WHO, 2006) as set out in **Table 5**.

Table 5: Specifications for chemical and microbiological purity of the food enzyme

Contaminant	Norm
Lead	Less than 5 mg/kg
Cadmium	Less than 0.5 mg/kg
Mercury	Less than 0.5 mg/kg
Arsenic	Less than 3 mg/kg
Aerobic microorganisms (30°C)	Less than 50,000 cfu/gram
Total coliforms	Not more than 30 cfu/gram
Escherichia coli	Absent in 25 grams
Salmonella sp.	Absent in 25 grams
Staphylococcus aureus	Absent in 1 gram
Clostridia sulphite reducing	Less than 30 cfu/gram
Antimicrobial activity	Not detected
Mycotoxins	No significant levels

With respect to mycotoxins (See JECFA specifications http://www.fao.org/docrep/pdf/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.

The proof that the food enzyme endo-inulinase complies with these specifications is shown by the analyses on various different batches provided in **Appendix 9.** The methods of analysis are also provided in Appendix 9.

6.1.3.1 Molecular Mass, Subunit Structure and Amino Acid Sequence of the Enzyme Protein

Regarding the subunit structure and amino acid sequence of the endo-inulinase protein from *Aspergillus oryzae*, it concerns a monomer containing a signal peptide. A signal peptide sequence is used for the secretion of the enzyme.

Further detail is provided under **Confidential Commercial Information (6.1.3)**

6.1.3.2 Information on post translational modification of the enzyme protein

The only post-translational modification that can be considered here is the cleavage of the signal peptide during the secretion of the enzyme. This cleavage is performed during the secretion of the enzyme. Via SDS-gel determination, it can be concluded that the Molecular Mass of the enzyme protein is correct. Further detail regarding the signal peptide and the amino acid sequence is provided under **Confidential Commercial Information (6.1.3).**

6.1.3.3 Information and rationale on protein engineering of the enzyme protein

Not applicable. Protein engineering has not taken place.

6.1.3.4 Information on side activities of the enzyme protein which might cause adverse effects

As far as the Applicant is aware, the endo-inulinase described in this dossier does not possess any enzymatic side activities which might cause adverse effects.

Microbial food enzymes are concentrates typically containing minor amounts of other enzyme activities (side activities) naturally produced by the microorganism. However, these activities are not relevant from an application or safety point of view.

Food enzymes are biological concentrates containing – apart from the desired enzyme protein (expressing the activity intended to perform a technological purpose in a certain food process, also called ‘main enzyme activity’) - some other organic substances. This is the reason why JECFA developed the TOS concept for food enzymes and why it is important that the source of a food enzyme is safe.

These other substances may include various enzyme activities (defined as ‘side activities’) derived from the producing microorganism and the fermentation medium. Like all living cells, microorganisms produce a variety of enzymes responsible for the hundreds of metabolic processes that sustain their life. As microorganisms do not possess a digestive system, microorganisms excrete the digestive enzymes into the medium in which they are growing in order to break-down substrates in that medium into smaller molecules (e.g. sugars), which they can then take up as food.

Most of these enzymes are hydrolases that digest carbohydrates, proteins and lipids. These are the very same activities that play a role in the digestion of food by – amongst others – the intestinal micro flora in the human body and in the production of fermented food.

The presence in food of enzyme activities and of the potential reaction products is natural and should not be of any safety concern. In addition, it is generally accepted that the enzyme proteins themselves do not pose any safety concern either.

During the production of food enzymes, the main enzyme activity is normally not separated from the other substances present. Consequently, the food enzyme may contain a number of other enzymes excreted by the microbial cells or derived from the fermentation medium. Other strains of *Aspergillus*

sp., selected to produce other main enzyme activities, will produce and excrete the same set of enzymatic activities, albeit in various amounts. Consequently, the food enzymes from *Aspergillus sp.* which are approved and used in food processes for many years (refer to Section 5.2.2), will also contain these activities. These activities are of no safety concern (refer to Section 6.1.3) and their fate in the final food will be the same as that of the main enzyme activity. If they also do not break down the main enzyme protein and do not play a role in the intended technological function in food processing, these side activities are not considered as 'significant' enzyme activities, and there is no reason to specifically investigate their nature.

'Significant' enzyme activities are considered those that do play a role in the intended food processes. In those cases where unwanted side activities are produced (e.g. that break down the main enzyme protein or that could create an unwanted technological effect during food processing), care is taken that such activities are not present in the food enzyme – either by genetic modification of the producing organism, or by specific purification steps. Otherwise, the customer/user would simply not use the food enzyme. In those cases where other activities are present that play a supportive (subsidiary) role in the intended technological function, their nature is investigated and will be reported in the respective dossiers.

In the case of the food enzyme described in this dossier, no other significant enzymatic activities have been identified.

6.1.3.5 Information on the activity of the food enzyme under various reaction conditions

The activity of the food enzyme endo-inulinase from *Aspergillus oryzae* was measured under various pH and temperature conditions using the analytical method provided in **Appendix CCI A1.** The results are presented in **Figures 2 & 3** below.

Figure 2: Relative endo-inulinase activity vs pH and temperature

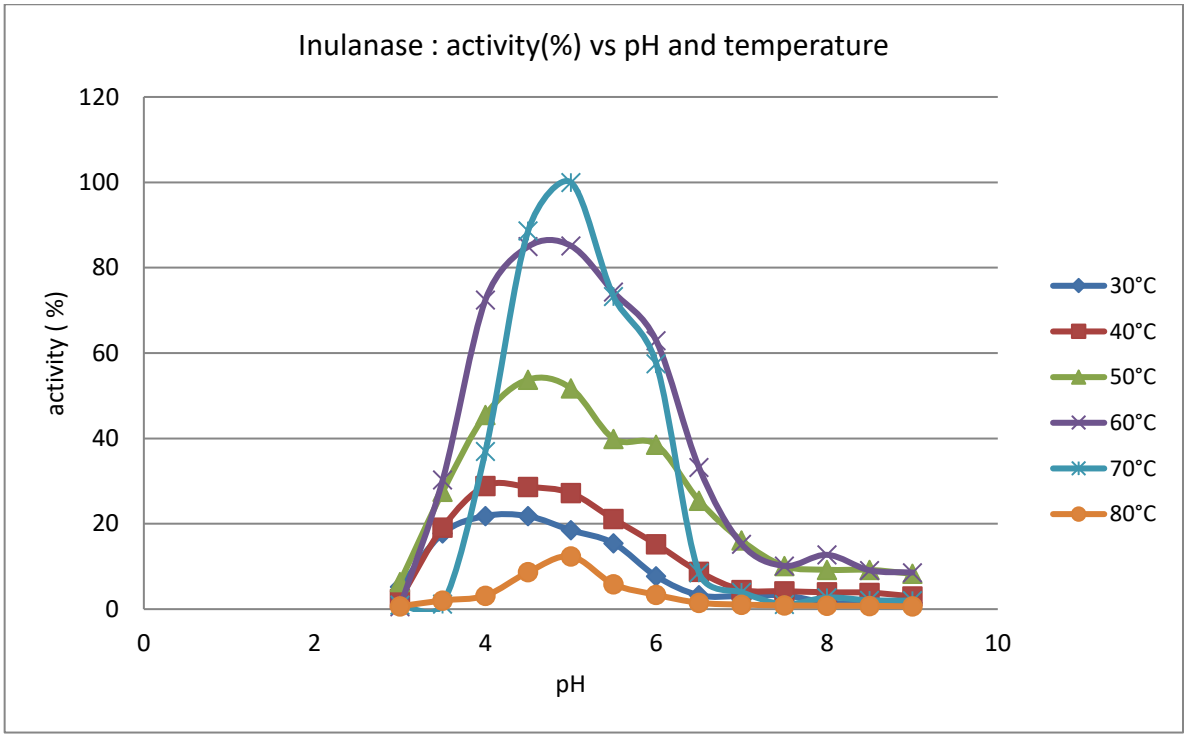
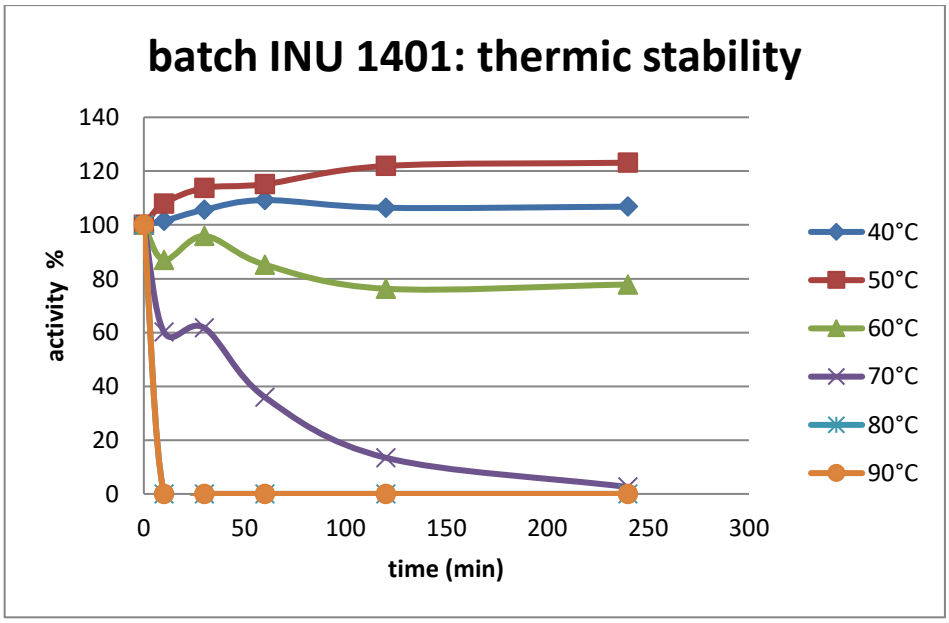


Figure 3: Relative endo-inulinase stability



As can be concluded from the given figures above, the food enzyme endo-inulinase from *Aspergillus oryzae* exhibits activity from pH 3.5 till pH 6.5, and from 40°C till 70°C. The optimum pH range lies between pH 4 and 6, whereas the optimum temperature range lays between 60 and 70°C. The endo-inulinase is not thermally stable above 70°C.

6.1.3.6 Data on the stability of the food enzyme during storage and before use

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

Tests of stability were performed on products at end of shelf-life and the results are provided in **Appendix 10** and in **Table 6.**

Table 6: Stability of Commercial Products

Batch	**Activity just after production** **(IU/ml)**	**Activity end of shelf life after 12 months** **(IU/ml)**
Oligofructase 3000 Batch: 0000224801	2633	2943
Oligofructase 3000 Batch: 0000311844	2887	2702
Oligofructase 3000 Batch: 0000311854	2777	2981

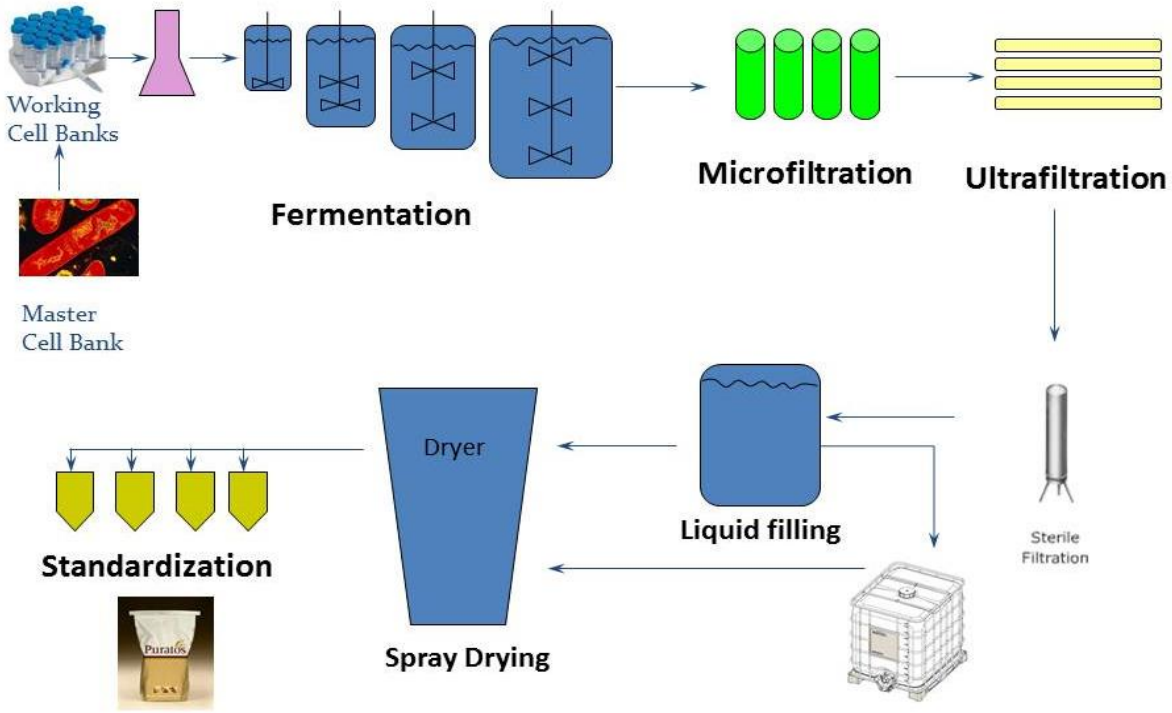
6.1.4 Manufacturing process

(As per section 3.3.2 A.4 of the Application Handbook as at 1 March 2016)

The fermentation and downstream processes are schematically represented in **Figure 4.** The production process is completely closed until the formulation of the commercial product.

Each fermentation run is started from pure starter cultures.

Figure 4: Schematic representation of the production process of the food enzyme



6.1.4.1 Description of the Process

Cultures are started on Petri dishes and then transferred to fermenters of increased volume up to the production fermenter of 100 m³. The fermenters used are designed for submerged culture with central stirrer.

The carbon source for the *Aspergillus oryzae* fermentation is chosen among e.g. the following: sucrose, maltose, glucose, maltodextrins and starch. The enzyme preparation does not contain any allergens which could come from the fermentation medium. If allergens are possibly present, the enzyme preparation will clearly mention these.

The nitrogen source is chosen among the e.g. following: peptones, protein hydrolysates, yeast extracts, glutamate and urea.

The medium is usually supplemented with various inorganic salts. A fed-batch is used to provide additional nutrients all along the fermentation. Chemicals used in the fermentation medium are all certified food grade by the suppliers. As the production of this food enzyme falls under the EU Food Hygiene Regulation, all raw materials used during fermentation and recovery are of food grade quality.

Specific process parameters are applied and controlled throughout the whole fermentation.

The plant is automated and is managed from the control room by an operator. All the crucial parameters are stored in production recipes and their actual values are recorded on-line and displayed on a screen.

The production strain is kept as pure culture in Master Cells Bank (MCB) with sterile glycerol and stored at -70°C. “Security” stocks of MCB are kept in different locations in the company. Based on those MCB, Working Cells Bank (WCB) are prepared. The purity is verified before use in production.

During fermentation samples are taken on a regular basis and analysed.

During production, the operator has the opportunity to adjust the parameters ensuring an optimal fermentation. The fermentation process is completely closed to avoid any contamination from outside (and to prevent any leakage from the vessels).

The fermentation ends when the optimal requested level of biomass and enzymatic activity is obtained.

At the end of the fermentation, samples are taken and analyzed for:

- total cell and viability count; and
- contaminants.

The Inulinase produced by *Aspergillus oryzae* described above is secreted in the culture medium. After fermentation and heat treatment, the biomass is separated from the enzyme containing culture medium by microfiltration.

At the end of the microfiltration process, the biomass is destroyed by an alkaline treatment (pH 12). The biomass can be then disposed in the waste water treatment station without any environmental risk. Control experiments have shown that no viable cells survived this treatment.

The enzyme preparation is concentrated by ultrafiltration before sterile filtration. The obtained solution is free from any microorganisms.

Further detail is provided under **Confidential Commercial Information (6.1.4)**

6.1.4.2 Good Manufacturing Practice and HACCP

The enzyme is manufactured according to good manufacturing practices (GMP) and the principals of HACCP. When manufactured in the EU, it is also subject to Regulation (EC) No 852/2004 -Food Hygiene Regulation.

A Certificate for Puratos Global Standard for Food Safety is provided as **Appendix 20** as supporting evidence for the implementation of GMP.

A HACCP plan is applied to the production of endo-inulinase to manage any potential risks that may come from fermentation**.**

The HACCP plan is provided with the **CCI material (6.1.4 Manufacturing Process).**

Potential Hazards

In order to comply with current GMP and HACCP principles for food production, the following potential hazards in food enzyme production are taken into account:

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 are controlled.

Microbiological Hygiene

For optimal enzyme production, it is important that hygienic conditions during the whole fermentation process are controlled. Microbial contamination would immediately result in less growth of the production organism and consequently in a low yield of the required enzyme protein and eventually a rejected product.

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.

Control Measures

The main measures to control the hazards identified above are:

Identity and Purity of the Producing Microorganism
During the preparation of the inoculum, several controls are performed on cultures of spores on Petri dishes.

The first control is visual. The spores of *Aspergillus oryzae* are green. Spores of other colours are tracked.

The liquid collected after cultures is controlled for the absence of bacteria using LB-nystatine.

The presence of yeast is also checked by incubation on YGC.

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

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

Chemical Contaminants
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-Process Testing and Monitoring
In addition to the above-mentioned control measures, in-process testing, and monitoring is performed to guarantee an optimal and efficient enzyme production process and a high-quality product.

These in-process controls comprise:

Microbial Controls
Absence of significant microbial contamination is analysed 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
Monitoring of fermentation parameters may include:

- pH;
- Temperature; and
- Dissolved oxygen content.

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.

6.1.5 Specification for identity and purity

(As per section 3.3.2 A.5 of the Application Handbook as at 1 March 2016)

The commercial enzyme product also complies with Standard 1.1.1 Structure of the Code and general provisions – 1.1.1 – 15(2) Identity and Purity and Schedule 3 – Identity and Purity - S3-2 - Substances with specifications in primary sources. The product complies with current versions of Food Chemicals Codex (10th ed) and JECFA specifications for chemical and microbiological purity of food enzymes (FAO/WHO, 2006).

Evidence that the food grade enzyme complies with these specifications is shown by the analyses on various different batches **(Appendix 8).**

The enzyme preparation does not contain any allergens which could come from the fermentation medium. If allergens are possibly present, the enzyme preparation will clearly mention these.

6.1.6 Analytical method for detection

(As per section 3.3.2 A.6 of the Application Handbook as at 1 March 2016)

The endo-inulinase enzyme preparation is to be used in the food industry as a processing aid. The Application handbook does not require this information in the case of an enzymatic processing aid.

6.2 Information on the Safety of an enzyme processing aid

(As per section 3.3.2 C of the Application Handbook as at 1 March 2016)

6.2.1 General information on the use of the enzyme as a food processing aid in other countries

(As per section 3.3.2 C.1 of the Application Handbook as at 1 March 2016)

Refer to overseas approvals in Sections 5.1 and 5.2

6.2.2 Information on the potential toxicity of the enzyme processing aid

(As per section 3.3.2 C.2 of the Application Handbook as at 1 March 2016)

6.2.2.1 Information on the Enzyme's prior history of human consumption

Refer to overseas approvals in Sections 5.1 and 5.2

6.2.2.2 Information on any significant similarity between the amino acid sequence of the enzyme and that of known protein toxins

The Applicant considers that approval and use of this enzyme in other countries as outlined in Sections 5.1 and 5.2 demonstrates a history of safe human consumption.

6.2.2.3 Assessment of genotoxicity

AMES test

A reverse mutation assay (AMES Test) using *Salmonella typhimurium* and *Escherichia coli* strains has been performed on the food enzyme.

The method was designed to meet the requirements on the OECD Guidelines Testing of Chemicals N°471 "Bacterial Reverse Mutation Assay" (OECD, 1997a) Methods B13/14 of Commission Directive 2000/32/EC and the USA, EPA (TSCA "bacterial reverse mutation test") OPPTS harmonised guidelines.

The results of the study are presented in **Appendix 11.** The AMES test was performed with a product named Oligo F300, which is the same product as for which approval is being sought. This is the same substance as will be used in food production, if approval is granted.

The assay used tester strains TA1535, TA 100, TA1537, TA102 and TA98 of *S. typhimurium*, selected to detect various types of mutagens. The test was performed with and without metabolic activation using an S9 activation system.

The dose range was determined in a preliminary toxicity assay and was 50 to 5000 µg/plate in the experiment. The experiment was repeated on a separate day using the same dose range, fresh cultures of the bacterial strains and fresh test material formulations.

The vehicle (sterile distilled water) control plates gave counts of revertant colonies within the normal range. All of the positive control chemicals used in the test induced marked increases in the frequency of revertant colonies, both with or without metabolic activation. Thus, the sensitivity of the assay and the efficacy of the S9-mix were validated.

The test material caused no visible reduction in the growth of the bacterial background lawn at any dose level. The test material was, therefore, tested up to the maximum recommended dose level of 5000 µg/plate. No test material precipitate was observed on the plates at any of the doses tested in either the presence or absence of S9-mix.

No significant increases in the frequency of revertant colonies were recorded for any of the bacterial strains, with any dose of the test material, either with or without metabolic activation.

The test material was considered to be non-mutagenic under the conditions of this test.

Chromosomal aberration test

A Chromosome aberration test in L5178Y TK+/- mouse lymphocytes has been performed with the enzyme preparation.

The method was designed to meet the requirements of the OECD Guidelines Testing of Chemicals N°487.

The results of the study are presented in **Appendix 12.**

After a preliminary toxicity test, the test item dissolved in water for injections was tested in two independent experiments, with and without a metabolic activation system, the S9 mix, prepared from a liver microsomal fraction (S9 fraction) of rats induced with Aroclor 1254.

Each treatment was coupled to an assessment of cytotoxicity at the same dose-levels. Cytotoxicity was evaluated by determining the PD (Population Doubling) of cells and quality of the cells on the slides has also been taken into account.

Since the test item was found non-severely cytotoxic in the preliminary test, the highest dose-level selected for the main experiments was 5000 µg/mL, according to the criteria specified in the international regulations. With one exception which was not considered to impact the validity of the results, the mean frequency of micronucleated cells for the vehicle control was as specified in the acceptance criteria and positive controls showed clear unequivocal increases in the frequency of micronucleated cells, the study was therefore considered to be valid.

No noteworthy toxicity was induced at any dose-levels following the 3- and 24-hour treatments.

The dose-levels selected for micronucleus analysis were 1250, 2500 and 5000 µg/mL for the 3- and 24-hour treatments, the latter being the highest recommended dose-level.

No significant increase in the frequency of micronucleated cells was noted after 3- and 24-hour treatments.

The test item did not induce any chromosome damage, or damage to the cell division apparatus, in cultured mammalian somatic cells, using L5178Y TK+/- mouse lymphoma cells, either in the presence or in the absence of a rat metabolizing system.

6.2.2.4 Sub-chronic oral toxicity test

Sub chronic repeated dose oral toxicity (2 weeks)

A preliminary sub chronic oral toxicity test was performed for two weeks in order to select dosages for a 13-week toxicity study.

The report of the study is presented under **Appendix 13.**

Groups of three male and three female CD rats received the food enzyme, by daily oral gavage administration, at dosages of 2750, 11000 or 27500 IU/kg/day for 14 days. A similarly constituted control group received the vehicle (water) alone. The formulations were administrated at a constant volume-dosage of 10.58 ml/kg/day. Each animal was examined daily for clinical signs. Body weight was recorded once before the beginning of the treatment period, on the first day of treatment and at least once a week until the end of the study. Food consumption was measured for each week of treatment. Each animal was killed on day 15 and subjected to a detailed necropsy.

There were no deaths and no signs related to treatment.

The bodyweight gains of treated and control animals were generally similar and therefore unaffected by treatment.

After 14 days of treatment there were no macroscopic findings attributed to treatment.

It is concluded that the administration of the inulinase at dosages up to 27500 IU/kg/day did not produce any toxic effect and was considered to be the no-observed-adverse-effect level (NOAEL) in this study.

Sub chronic repeated dose oral toxicity (13 weeks)

A sub chronic oral toxicity test was performed in order to evaluate the potential toxicity of the food enzyme following daily oral administration to rats for 13 weeks.

The report of the study is presented under **Appendix 14.**

The study design was designed to meet the requirements of OECD (1981) Guidelines No 408 (OECD, 1998).

Groups of ten male and ten female CD rats received the enzyme at dosages of 2750/3134, 11000/12508 or 27500/31285 IU/kg/day by daily oral gavage administration for the duration of the studies. For each dosage, two numbers are mentioned. This is due to the use of two different batches during the test which were dosed at different levels. When it was necessary to restock the lab with the enzyme, the fact that the new batch was higher concentrated was not considered as problematic for the result of the test.

A control group received the vehicle (water) alone.

There were no premature deaths or unscheduled sacrifices during the study.

There were no test item treatment-related clinical signs. No changes were observed at the Functional Observation Battery.

The mean body weight was unaffected by the test item treatment and food intakes were similar in test item-treated animals when compared with controls.

There were no findings at the ophthalmology examination at the end of the treatment period.

There were no test item treatment-related findings at hematology and blood biochemistry investigations.

At histopathology, there was a higher incidence of mucification of the vaginal epithelium in females given 27500 IU/kg/day. This finding was considered to be non-adverse.

Under the experimental conditions of this study, the No Observable Adverse Effect Level (NOAEL) was considered to be 27500 IU/kg/day.

6.2.2.5 Respiratory Toxicity

No allergenicity has been noticed during the R&D works, or during pre-industrial trials and industrial up scaling, nor during downstream processing. Enzyme preparations are regarded as respiratory sensitisers (R42/H334). As such, measures should be taken to minimize the inhalation exposure of workers and inhalation toxicity studies are thus normally not required. No dust formation can occur with this liquid enzyme preparation and based on the inhalation study on rats with a liquid sample of the food it can be considered safe under its normal condition of use.

An acute inhalation study has been performed (**Appendix 15**) in compliance with the OECD guidelines for testing chemicals N° 403 (OECD, 2009). The inhalation toxicity was performed on 10 rats exposed during 4 hours to an aerosol.

No death occurred in the group of ten rats exposed to the maximal attainable concentration of 3.78 mg/L for four hours. The acute inhalation median lethal concentration (4hr LC50) of the enzyme was therefore considered to be greater than 3.78 mg/L.

6.2.2.6 Dermal irritation

An acute dermal irritation test has been performed (refer to **Appendix 16**) in compliance with OECD principles GLP and directive 2004/10.

This acute dermal irritation test in the rabbit was performed with the food enzyme on three animals. The potential for inflammatory or corrosive activity of the enzymes to skin was assessed by a single exposure to 0.5-ml material for four hours. Responses were noticed after 1, 24, 4, 72 hours and reported.

The enzyme can be classified as non-irritant to skin.

6.2.2.7 Eye irritation

An acute eye irritation test has been performed (refer to **Appendix 17**) in compliance with OECD principles GLP and directive 2004/10.

This acute eye irritation in the rabbit was performed with 0.1 ml of food enzyme on three animals. The consequences on the eye were looked at 1, 24, 48 and 72 hours after treatment.

The enzyme can be classified as non-irritant to the eye.

6.2.2.8 Test material used in genotoxicity and sub-chronic toxicity studies

Enzyme preparations used in the various toxicological tests are representative of the food enzyme to be used.

All individual safety studies and reporting of data have been performed according to the respective OECD guidelines. For further details, see the individual studies.

Table 7: Composition and specifications of the test materials

TEST	**Ames**	**Micronucleus assay** **- 14 days** **- 90 days** **- dermal irritation** **- eye irritation**	**90 days** **- inhalation test**
Batch no	B01/2002	1203	1208
Ash (%)		0.12	0.15
Water (%)		99.26	99.33
TOS (%)		0.62	0.52
Activity (IU/ml)	2344	2598	2957
IU/mg TOS		419	568

Certificates of Analysis are given in **Appendix 18.**

Summary of the toxicological data

The food enzyme has been subjected to a standard package of toxicological tests, with the following results:

- Ames test: no mutagenic activity under the given test conditions
- Chromosomal aberrations: no clastogenic activity under the given test conditions
- 90-day oral toxicity on rats: The No Observed Adverse Effect Level (NOAEL) of at least 27500 IU/kg/day or 189.65 mg TOS/kg/day (based on 145.55 Units/mg TOS, Table 4).

Calculation of TMDI

Endo-inulinase from *Aspergillus oryzae* is used to produce fructo-oligosaccharide (FOS). This FOS is also used in the manufacture of a wide variety of foods. Due to this wide variety of applications, the most appropriate way to estimate the human consumption in the case of food enzymes is using the so-called Budget Method (Hansen, 1966; Douglass *et al.*, 1997). This method enables 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 consumption of the FOS is difficult to evaluate but according to Coussement 1999, we could consider 20g/day as an extreme consumption.

For the calculation of the TMDI, the maximum use level is chosen. In this specific application, we will assume that all the TOS goes into the final product.

Table 08: Determination of maximum level

Application	Raw material (RM)	Maximal recommended use level (IU/kg RM)	Maximal recommended use level (mg TOS/kg RM)	Final food	Ratio RM/final food	Maximal level in final food (IU/kg food)	Maximal level in final food (mg TOS/kg food)
FOS production	inulin	2750	18.9	FOS	1.1	3025	20.86

The Total TMDI can be calculated on basis of the **maximal** values found in food, multiplied by the average consumption of food/kg body weight/day. Consequently, the Total TMDI will be:

Table 09: TMDI

TMDI in food (mg TOS/kg body weight/day)	Total TMDI (mg TOS/kg body weight/day)
20.86*(0.02/60)	**0.0069**

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

- It is assumed that ALL producers of the above-mentioned foodstuffs use the specific enzyme endo-inulinase from *Aspergillus oryzae*;
- It is assumed that ALL producers apply the HIGHEST use level per application;
- For the calculation of the TMDI's in food, only the highest theoretical amount of TOS were 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;

The Margin of Safety (MoS) for human consumption can be calculated by dividing the NOAEL derived from the 90-day oral toxicity study by the Total Theoretical Maximal Daily Intake (TMDI). The TMDI of the food enzyme is 0.0069mg TOS/kg body weight/day. Consequently, the Margin of Safety is:

MoS = 189.65/0.0069= 27485.5

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. Consequently, there are no safety reasons for laying down maximum levels of use.

6.2.3 Information on the potential allergenicity of the enzyme processing aid

(As per section 3.3.2 C.3 of the Application Handbook as at 1 March 2016)

Intake Allergies

The amino-acid sequence for the endo-inulinase enzyme protein has been determined, as described in Section 6.1.3 and 6.1.3 CCI. Therefore, the homology search as described below of the EFSA CEF Guidance document on food enzymes (EFSA, 2009) has been performed.

The online tool used to search allergens database was http://www.allergenonline.org/ and the sequence without signal peptide as query was used (refer to **Appendix CCI A4**). Based on Full Fasta search method, Sliding 80mer Window search method or 8mer Extract Match search method significant similarities of endo-inulinase from *Aspergillus oryzae* with 2 sequences of known allergenic proteins have been detected with the 80mer search but no similarities were detected with 8mer.

The two sequences identified by the 80mer search are linked to the same article. According to the NCBI web reference mentioned in allergenonline.org, the article used for the inclusion of the sequences on the database was not published.

Those two sequences are linked to the tomato (*Solanum lycopersicum*) and described as “minor” allergens. The tomato is not recognised by European legislation as crucial source of allergens in food. It is not mandatory to declare in labelling the presence of tomato in a transformed food. Tomato is largely used in many different kinds of food all around Europe. This large presence of tomato in European food and the fact that the European Commission did not take any measure against tomato in its strategy for the information regarding allergens, permits a conclusion that the similarity reported in the database of allergens will not pose any problems for consumers.

Because they are proteins, enzymes could theoretically have the potential to cause allergic responses. However, 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 (Dauvrin *et al.*, 1998). The overall conclusion was that – as opposed to exposure by inhalation – there are no scientific indications that the small amounts of enzymes in food can sensitize or induce allergy reactions in consumers.
- Enzymes when used as digestive aids are ingested daily, over many years, at much higher amounts when compared to enzymes present in food (up to 1 million times more). Wüthrich (1996) published a list of enzymes used as digestive aids and concluded that they are not potent allergens by ingestion.

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 assumption 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 enzyme proteins used in food being homologous to known food allergens[4].
- 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, the tertiary conformation of the enzyme molecule is destroyed. In general, these alterations in conformation are associated with decrease in

4 The only enzyme protein used in food an known to have a weak allergenic potential is egg lysozyme

the antigenic reactivity in humans: in the vast majority of investigated cases, denatured proteins are much less immunogenic than the corresponding native proteins (Valenta and Kraft, 2002; Valenta, 2002; Takai *et al.*, 1997; Takai *et al.*, 2000; Nakazawa *et al.*, 2005; Kikuchi *et al.*, 2006).

- In addition, residual enzyme proteins 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 (FAO/WHO, 2001; Goodman *et al.,* 2008).
- 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.

Respiratory Allergies

No allergenicity has been noticed during the R&D works, or during pre-industrial trials and industrial up scaling, nor during downstream processing.

Enzyme preparations are regarded as respiratory sensitisers (R42). As such, measures should be taken to minimize the inhalation exposure of workers and inhalation toxicity studies are thus normally not required.

No dust formation can occur with this liquid enzyme preparation and based on the inhalation study on rats with a liquid sample of the food it can be considered safe under its normal condition of use.

An acute inhalation study has been performed (refer to Appendix 14) in compliance with the OECD guidelines for testing chemicals N° 403 (OECD, 2009).

The inhalation toxicity was performed on 10 rats exposed during 4 hours to an aerosol.

No death occurred in the group of ten rats exposed to the maximal attainable concentration of 3.78 mg/L for four hours. The acute inhalation median lethal concentration (4hr LC50) of the enzyme was therefore considered to be greater than 3.78 mg/L.

6.2.4 Safety assessment reports prepared by international agencies or other national government agencies, if available

(As per section 3.3.2 C.4 of the Application Handbook as at 1 March 2016)

The food enzyme endo-inulinase has been evaluated to be safe via the GRAS system in the USA, where it can be used on the market and can be legally used in most of the EU. Moreover, the food enzyme has been submitted for evaluation to the JECFA and to the EFSA.

6.3 ADDITIONAL INFORMATION RELATED TO THE SAFETY OF AN ENZYME PROCESSING AID DERIVED FROM A MICROORGANISM

(As per section 3.3.2 D of the Application Handbook as at 1 March 2016)

6.3.1 Information on the source microorganism

(As per section 3.3.2 D.1 of the Application Handbook as at 1 March 2016)

The microorganism that is used for the production of endo-inulinase, is the *Aspergillus oryzae*.

A. oryzae tend to prefer environments that are rich in oxygen, as they are molds that inhabit the surface of various substrates that provide beneficial nutrients to them. They also prefer environments between 30 and 40 degrees Celsius that have adequate moisture. *A. oryzae* are a domesticated species and are most commonly found in northern regions, specifically in East Asia, but can be found anywhere. The Aspergillus genus is extremely common, although *A.oryzae* specifically is more rare due to its domestication for use in fermentation in the food industry. *A. oryzae* has been determined to be relatively safe for use in food processing because of its domestication and evolution from wild-type relatives *A. flavus* and *A.niger*, which led to an inactivation the proteins that code for its toxin pathway. As A. oryzae is a domesticated fungus native to humid East Asian regions, it is a microorganism that is primarily used in Japanese and Chinese food production.

Aspergillus oryzae is deposited under the number MUCL- 44346 at the Belgian co-ordinated collections of microorganisms located at the University of Louvain-la-Neuve.

The address of the Culture Collection is:

Mycothèque de l'Université catholique de Louvain

Croix du Sud 2, box L7.05.06
B-1348 Louvain-la-Neuve, Belgium

According to the current state of the art, the taxonomic classification of this microorganism is as follows:

Genus:	*Aspergillus*
Species:	*Aspergillus oryzae*
Synonyms[5]:	*Aspergillus flavus var. oryzae*

6.3.2 Information on the pathogenicity and toxicity of the source microorganism

(As per section 3.3.2 D.2 of the Application Handbook as at 1 March 2016)

Aspergillus oryzae does not appear on the list of pathogens in Annex III of Directive 2000/54/EC on the protection of workers from risks related to exposure to biological agent at work, as it is globally regarded as a safe microorganism:

- In the USA, *Aspergillus oryzae* is listed as a Class 1 Containment Agent under the National Institute of Health (NIH) Guidelines for Recombinant DNA Molecules (NIH, 1994). Class 1 Containment Agents are microorganisms with the lowest safety concern, such as baker's yeast;
- The US EPA has exempted *Aspergillus oryzae* from review by the Agency, due to its extensive history of safe use (EPA, 1997);
- In Europe, *Aspergillus oryzae* is classified as a low-risk-class microorganism, as exemplified in the listing as Risk Group 1 in the microorganism classification lists of the German Federal Institute for Occupational Safety and Health (BauA, 2010). It is not mentioned on the list of pathogens in Belgium (Belgian Biosafety Server, 2010).

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

The strain does not produce toxicologically significant amounts of mycotoxins – this information has been provided in **Appendix 9.**

6.3.3 Information on the genetic stability of the source organism

(As per section 3.3.2 D.3 of the Application Handbook as at 1 March 2016)

This information is provided in Sections 6.4.1.3 and 6.4.1.4.

5 Reference: Mycobank taxonomic database (see: http://www.mycobank.org/Biolomics.aspx?Table=Mycobank&Page=200&ViewMode=Basic). More synonyms can be found under each of the names mentioned.

6.4 ADDITIONAL INFORMATION RELATED TO THE SAFETY OF AN ENZYME PROCESSING AID DERIVED FROM A GENETICALLY-MODIFIED MICROORGANISM

(As per section 3.3.2 E of the Application Handbook as at 1 March 2016)

6.4.1 Information on the methods used in the genetic modification of the source organism

(As per section 3.3.2 E.1 of the Application Handbook as at 1 March 2016)

6.4.1.1 Characteristics of the Recipient or (when appropriate) Parental Organism

Phenotypic and Genetic Markers

EPA (EPA 1997) mentions that *Aspergillus oryzae* is a member of the *A. flavus* group of *Aspergillus* species. The *A. flavus* group, which also now includes *A. sojae, A. nomius* and *A. parasiticus* (see below) is defined by the production of spore chains in radiating heads which range in color from yellow-green to olive brown. The conidiophores are roughened and colorless. The spores themselves have conspicuous ridges and echinulations (spines). Sclerotia are occasionally produced. *A. oryzae/flavus* species have never been connected to a sexual or teleomorphic stage. However, the teleomorphic stages of other *Aspergillus* species have been demonstrated by the formation of cleistothecia. These species belong to the genera *Emericella, Neosartorya* and *Eurotium,* all belonging to the ascomycetous family *Eurotiaceae*. Either the sexual stages of the *A. flavus* group have not been recognized as such, being identified as completely different species based on morphology, or this group of fungi are "degenerate", having lost the ability to form sexual spores and mycelia.

A. oryzae is considered to be a domesticated variant of *A. flavus* (Kurtzman et al. 1986). Through long-time use, *A. oryzae* strains seem to have been selected to exhibit reduced sporulation, have more aerial mycelia and exhibit no environmental survival structures like sclerotia or the presence of aflatoxins that might function to inhibit grazing by insects. These morphological features that differentiate *A. oryzae* from *A. flavus* may represent adaptations to the artificial culture conditions of the koji fermentation. Misidentification of new isolates not obtained from well-established cultures is always a possibility, since the key morphological differences between the two species seem related to culture adaptation. However, the source of *A. oryzae* strains for industrial fermentations today is likely to be standard culture collections. Environmental isolates of aspergilli would likely be identified as *A*. *flavus* rather than the laboratory-adapted *A*. *oryzae.*

Degree of Relatedness between Recipient and Donor(s)

The recipient and donor organisms are not of the same species. Information regarding the donor species can be found in the CCI information in Section 6.4.1.2.

Description of Identification and Detection Techniques

In order to demonstrate the presence/absence of the production strain in the enzyme preparation, a sample can be grown in 500 ml Aspergillus Minimum Medium (AMM) containing 70mM NaNO3, 10 g/l glucose and salts (11mM KH2PO4, 2mM MgSO4, 7mM KCL and trace elements) and incubated

at 30°C, 200 rpm for 16 h.) Alternatively, standard methods for the detection of *Aspergillus oryzae* can be used to identify the production organism.

Source and Natural Habitat of the Parental Microorganism

A. oryzae is considered to be a domesticated variant of *A. flavus* (Kurtzman et al. 1986). Through long-time use, *A. oryzae* strains seem to have been selected to exhibit reduced sporulation, have more aerial mycelia and exhibit no environmental survival structures like sclerotia or the presence of aflatoxins that might function to inhibit grazing by insects. These morphological features that differentiate *A. oryzae* from *A. flavus* may represent adaptations to the artificial culture conditions of the koji fermentation. The source of *A. Oryzae* strains for industrial fermentations today is likely to be standard culture collections. Environmental isolates of Aspergilli would likely be identified as *A. flavus* rather than the laboratory-adapted *A. oryzae*.

Organism with Which Transfer of Genetic Material is known to occur under Natural Conditions and Presence of Indigenous Genetic Mobile Elements

A. oryzae is considered to be a domesticated variant of *A. flavus* (Kurtzman et al. 1986). Through long-time use, *A. oryzae* strains seem to have been selected to exhibit reduced sporulation, have more aerial mycelia and exhibit no environmental survival structures. None of the plasmids used is carrying any functional element for autonomous replication in Aspergillus species they are considered as plasmid shuttle vectors. The transformed DNA from these plasmids must therefore be integrated in the chromosomal DNA for remaining in the cells throughout generations/multiplications.

Information on the Genetic Stability of the Recipient Microorganism

A comparison of ITS and 18S sequences was performed between the mother strain and the production strain showed no differences.

Further specific detail is provided in the CCI document – 6.4.1.

Pathogenicity, Ecological and Physiological Traits

Pathogenicity is addressed in Section 6.3.2

Secondary metabolites

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 nutrient or low/high temperature) or defence mechanisms (e.g. production of antibiotic, antiviral or neurotoxic molecules). Biosynthesis of stress and/or defence secondary metabolites of toxicological relevance by industrial microbial production organisms during the fermentation process is thus highly unexpected (Sanchez and Demain 2002) and is furthermore avoided from an economical perspective to optimize production.

Aspergillus oryzae strains are being safely used for decades to produce Koji in Japan. In 1978, JECFA (http://whqlibdoc.who.int/trs/WHO_TRS_617.pdf, page 9) nevertheless expressed some reservations regarding food enzymes derived from non-pathogenic microorganisms commonly found as contaminants of foods, such as *Aspergillus niger,* and considered it necessary to establish purity specifications and to conduct short term toxicity experiments on such food enzymes. Because it was recognized that some strains of fungal origin, such as *Aspergillus oryzae*, might be able to produce certain secondary metabolites of potential safety concern, JECFA recommended testing food enzymes derived from fungal origin for the presence of the secondary metabolites such as aflatoxin B1, ochratoxin A, sterigmatocystin, T-2 toxin and zearalenone.

Based on the JECFA recommendations, for decades a wide variety of food enzymes from *Aspergillus sp.* have been tested for the presence of potential unsafe secondary metabolites. More recently, JECFA (http://www.fao.org/docrep/pdf/009/a0675e/a0675e00.pdf, page 64) slightly changed their recommendation by stating that food enzymes from fungal origin should not contain toxicologically significant levels of secondary metabolites that theoretically could be produced by the species in question. Therefore, food enzymes from *Aspergillus oryzae* should be tested for the specific secondary metabolites that could theoretically be produced by the species i.e. Aflatoxin.

The toxicological tests (refer to Section 6) and analyses (refer to Section 6) performed on the endo-inulinase produced by *Aspergillus oryzae* merely confirm the assumption of non-toxicity.

Description of History of Use

Aspergillus oryzae has been used for nearly 500 years in Japan to produce "Koji". The Koji is one of the bases of the traditional food in this country. It's essential for the production of miso (soybean paste), shoyu (soy sauce) and sake (rice wine). Koji, the culture of koji mold on soy, rice or wheat is mixed with substrate and together they are fermented to produce foods (Nojiro 1984).

During the preparation of Koji, it's the production of natural enzymes which interest the producer.

Enzymes began to be widely employed in industry in the middle of the twentieth century. Early products were of animal origin, such as bovine trypsin for leather tanning and pancreatic amylase for desizing (removal of size or sizing agents, generally starch, from warp yarns prior to weaving, to protect them against the abrasive action of loom parts). Shortly thereafter, the pancreatic amylase

for desizing was replaced by bacterial amylase. The amylase from *Aspergillus oryzae* was also applied in the baking industry, where it was added to wheat flour to increase the volume of bread made.

In the mid-80's gene technology began to be applied in industry, including industrial enzyme production. Because of its safe history and high capacity to produce various enzymes, *Aspergillus oryzae* was chosen to be a host organism to produce industrial enzymes.

History of Previous Genetic Modification

Obtaining of the host strain

The mother strain was obtained through the MUCL. No genetic modification has been performed on the mother strain.

Further specific detail is provided in the CCI document.

6.4.1.2 Characteristics of the Origin of the Inserted Sequences (Donor Organism)

DNA from Defined Donor Organisms

The DNA was cloned via PCR after the genomic DNA was isolated from the donor strain.

Further specific detail is provided in the CCI document.

Synthetic DNA

Not relevant

Nucleic Acids directly extracted from environmental sample

Not relevant

6.4.1.3 Description of the Genetic Modification

Characteristic of the Vector

The producing strain was obtained after a co-transformation of two plasmids. The first plasmid contains the active endo-inulinase gene for the production of the enzyme whereas the second plasmid is used as selection marker by providing the ability to the transformed strain to grow on a minimal medium. As none of the plasmids is carrying any functional element for autonomous replication the transformed DNA from these plasmids must therefore be integrated in the chromosomal DNA for remaining in the cells.

Further specific detail is provided in the CCI document.

6.4.1.4 Information related to the GMM

The source material for the food enzyme is *Aspergillus oryzae*. The organism has been genetically modified to produce the enzyme.

Specific detail is provided in the CCI document.

Rate and Level of Expression of the New Genetic Material and Activity of the Expressed Proteins

The best way to quantify the expression level of the inserted genetic material is to quantity the activity of the enzyme produced. The level of activity, the method of analysis and the mode of action of the enzyme are described in 6.1.3.

Description of Identification and Detection Techniques

The techniques used for identification and detection of the inserted sequences are presented under 6.4.1.3 and corresponding Southern blot hybridization results are presented under 6.4.1.4. The techniques for detection and verification of absence of the vector in the GMM are presented under 6.4.1.3.

Information on the Ability to Transfer Genetic Material to Other Organisms

A. oryzae is considered to be a domesticated variant of *A. flavus* (Kurtzman et al. 1986). Through long-time use, *A. oryzae* strains seem to have been selected to exhibit reduced sporulation, have more aerial mycelia and exhibit no environmental survival structures. None of the plasmids used is carrying any functional element for autonomous replication in Aspergillus species they are considered as plasmid shuttle vectors. The transformed DNA from these plasmids must therefore be integrated in the chromosomal DNA for remaining in the cells throughout generations/multiplications.

History of Previous Uses or Environmental Release of the GMM

The GMM has never been released in the environment. The absence of the GMM in the final product is demonstrated in Section 6.4.2.2.

Safety for Humans and Animals

Aspergillus oryzae does not appear on the list of pathogens in Annex III of Directive 2000/54/EC on the protection of workers from risks related to exposure to biological agent at work, as it is globally regarded as a safe microorganism:

- In the USA, *Aspergillus oryzae* is listed as a Class 1 Containment Agent under the National Institute of Health (NIH) Guidelines for Recombinant DNA Molecules (NIH, 1994). Class 1 Containment Agents are microorganisms with the lowest safety concern, such as baker's yeast;

- The US EPA has exempted *Aspergillus oryzae* from review by the Agency, due to its extensive history of safe use (EPA, 1997);

• In Europe, *Aspergillus oryzae* is classified as a low-risk-class microorganism, as exemplified in the listing as Risk Group 1 in the microorganism classification lists of the German Federal Institute for Occupational Safety and Health (BauA, 2010) It is not mentioned on the list of pathogens in Belgium (Belgian Biosafety Server, 2010).

As a result, *Aspergillus oryzae* can be used under the lowest containment level at large scale, GILSP, as defined by OECD (1992). *Aspergillus oryzae* is a class 1 microorganism. There is no indication that the modification changes anything regarding the safety. The absence of mycotoxins in production batches has been shown.

6.4.2 Information related to the product

6.4.2.1 Information Related to the Production Process

See Section 6.1.4.

6.4.2.2 Information Related to the Product Preparation Process

Demonstration of the Absence of the GMM in the Product

This is discussed under Description of Identification and Detection Techniques (6.4.1.1).

Information on the Inactivation of the GMM Cells and Evaluation of the Presence of Remaining Physically Intact Cells

As explained under 6.4.1, a sterile filtration step at the end of the purification process excludes the presence of remaining physically intact cells of the production microorganisms. This is validated by the demonstration of the absence of the GMM in the product (see under Section 6.4.2.2).

Information on the Possible Presence of Recombinant DNA

Absence of antibiotic resistance genes, absence of GMM and rDNA in the final product are demonstrated.

Further information is provided under CCI (6.4.2)

6.5 INFORMATION RELATED TO THE DIETARY EXPOSURE TO THE PROCESSING AID

(As per section 3.3.2 F of the Application Handbook as at 1 March 2016)

6.5.1 Dietary Exposure

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-inulinase 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 table below shows the maximal recommended use level for the food enzyme.

Table 10: Maximal Recommended Enzyme Use Levels

Application	Raw material (RM)	Recommended use levels (mU/kg RM)	Maximal recommended use levels (mU/kg RM)
FOS Production	Inulin	2250-3000	3000

As general practice the addition of enzyme is around 2500 IU/kg Raw Material. Depending on initial inulin chain length this may be a little bit more or less. Recommended use level would range from 2250 IU/kg Raw Material to a maximum recommended use level of 3000 IU/kg Raw Material.

Endo-inulinase from *Aspergillus oryzae* is used to produce fructo-oligosaccharide (FOS). This FOS is also used in the manufacture of a wide variety of foods for purposes such as supplementing with dietary fibre, sugar-reduction, bulking and texturizing properties. Due to this wide variety of applications, the most appropriate way to estimate the human consumption in the case of food enzymes is using the so-called Budget Method (Hansen, 1966; Douglass *et al.*, 1997).

The daily per capita intake can be estimated to range from 1-10 gram /day, depending on geographic and other parameters, based on van Loo et al (1995). This value seems reasonable since generally one can consider that recommended daily intakes for fibre are 25-35 gram and soluble fibres (such as FOS) may form a third of this intake. The specific theoretical maximum dietary exposure as well as the Margin of Safety have been calculated in section 6.2.2.8.

It had been estimated that American diets, which generally are low in dietary fibre, provide on average 2.5 g of oligofructose (plus 2.6 g inulin) from whole foods; the intake varied by gender and age, ranging from 1.3 g for young children to 3.5 g for teenage boys and adult males (Moshfegh et

al 1999). However, these estimates are decades ago and with recommendations for dietary fibre to increase, higher values can be expected currently; for example, in Europe FOS are applied in infant formula 4 g/d, milk powders 2–3 g/250 ml serving, infant cereals, dietary supplements 4 g/serving 5–20 g/d, besides cereal bars and bakery (Stephen et al 2017). Foods have also been supplemented with dietary fibre in the last decade, e.g. snack bars (3-5 g/ bar), breads, sugar-reduced bakery, ice cream, yoghurt, meal replacers, amongst others, with 2-5gram doses (Coussement 1999), and thus the level is likely to be increased; 20 gram /day in 2-4 doses should be considered as a high-end consumption.

The chicory FOS is also aimed at infant- and follow-on milk powders / formulae, and baby foods like growing-up-milks (toddler milks), biscuits for babies and toddlers, including ready-to-serve and dry.

Chicory oligofructose was determined as GRAS for its use in infant formula at a maximum level of 3 g/L formula as consumed and subsequently received a "no question" letter from the FDA in 2012 for GRN 392; this was further confirmed for chicory FOS/ oligofructose in GRN 576. The production of this chicory FOS is equivalent to that of above scheme. Standard 2.9.1 – Infant formula product permits the use of inulin-type fructans in infant formulae and follow-on formulae taking into account both the naturally occurring and added substances[6].

A summary of the foods likely to contain FOS is set out in **Table 9** noting that the enzyme itself is not present in the final food product containing FOS.

6 https://www.legislation.gov.au/Details/F2017C00332

6.5.2 A list of foods or food groups likely to contain the processing aid or its metabolites

(As per section 3.3.2 F.1 of the Application Handbook as at 1 March 2016)

Table 11: Foods Groups and Foods likely to contain the enzyme

Food Group	Food	Schedule 15 (S15-5)
1 Dairy Products (excluding butter and butter fat)	Milk and flavoured milks Yoghurt	1.1.2 - Liquid milk products and flavoured liquid milk 1.2.2 Fermented milk products and rennetted milk products
3 Ice cream and edible ices	Ice cream	
4 Fruits and vegetables	Mashed potato - prepared or in frozen meals (excluding *dry* mix types)	4.3 Processed fruits and vegetables
5 Confectionary	Candy - hard and soft	5.2 Sugar confectionary
6 Cereals and cereal products	Unbaked cereal goods - pasta, noodles and snack goods RTE breakfast cereals Breakfast bars, granola bars, energy bars, and diet/meal replacement bars	6.3 Processed cereal and meal products 6.4 Flour products (including noodles and pasta)
7 Breads and bakery products	Breads, biscuits, steamed bread, cakes, pancakes, tortillas, wafers, waffles, snack crackers	7.1 Breads and related products 7.2 Biscuits, cakes and pastries
11 Sugars, honey and related products	Pancake syrup	11.2 Sugars and sugar syrups
13. Special purpose foods	Infant formula & Follow on formula Infant cereals Meal replacement beverages and meal supplement beverages, including ready-to-drink beverages and dry beverage mixes	13.1 Infant formula products 13.2 Foods for Infants 13.3 & 13.4

20 Foods not included in items 0 to 14	Pudding mix, Mousse	20.2.0.3 Dairy and fat based desserts, dips and snacks
20 Foods not included in items 0 to 14	Salad dressings, sauces including cheese, Hollandaise, pasta	20.2.0.4 Sauces and toppings
20 Foods not included in items 0 to 14	Soups, *dry*	20.2.0.5 Soup bases
Other food types	Fillings used in pasta, such as tortellini, ravioli; Topped pizza	

6.5.3 The levels of residues of the processing aid or its metabolites for each food or food group

(As per section 3.3.2 F.2 of the Application Handbook as at 1 March 2016)

Maximal recommended use levels are set out in **Table 8.**

6.5.4 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

(As per section 3.3.2 F.3 of the Application Handbook as at 1 March 2016)

Not applicable

6.5.5 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

(As per section 3.3.2 F.4 of the Application Handbook as at 1 March 2016)

The safety has taken 100% market share as number, but practically a market share of 20% would already be considered very favorably.

6.5.6 Information relating to the levels of residues in foods in other countries

(As per section 3.3.2 F.5 of the Application Handbook as at 1 March 2016)

The active enzyme is exclusively used for the production FOS but consequently this active enzyme is not eliminated from the final product. The added enzyme is therefore remaining in the final food, but as we have shown there is no *active* enzyme present after the baking process, as the enzyme is denatured due to the high temperatures. The amount of recommended inulinase addition in all countries where the enzyme is sold is the same

6.5.7 For foods where consumption has changed in recent years, information on likely current food consumption

(As per section 3.3.2 F.6 of the Application Handbook as at 1 March 2016)

Not applicable.

REFERENCES

Arrêté du 19 octobre 2006 relatif à l'emploi d'auxiliaires technologiques dans la fabrication de certaines denrées alimentaires. http://www.legifrance.gouv.fr/affichTexte.do;jsessionid=C8C7772AB2563855B331A28AD00728B6.tpdjo09v_1?cidTexte=LEGITEXT000020667468&dateTexte=20130508, last visited on 8 May 2013

BauA (2010) Bundesanstalt für Arbeitsschutz une Arbeitsmedizin. Classification of Prokaryotes (Bacteria and Archaea) into Risk Groups. Technical Rules for Biological Agents (TRBA) 466.

Belgian Biosafety Server (2010). http://www.biosafety.be/RA/Class/ClassBEL.html, last visited on 14 August 2018.

Bindslev-Jensen C, Stahl Skov P, Roggen EL, Hvass P and Sidelmann Brinch D (2006). Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry. Food and Chemical Toxicology, 44, 1909–1915

Coussement P. (1999) Inulin and Oligofructose: Safe Intakes and Legal Status. J Nutr. 1999 Jul;129(7 Suppl):1412S-7S

Dauvrin T, Groot G, Maurer K-H, de Rijke D, Ryssov-Nielsen H, Simonse M and Sorensen TB (1998). Working group on consumer allergy risk from enzyme residues in food. An Amfep expert group evaluation study

Douglass JS, Barraj LM, Tennant DR, Long WR and Chaisson CF (1997). Evaluation of the Budget Method for screening food additive intakes. Food Additives and Contaminants, 14, 791-802

Earl, A. M., Losick, R. and Kolter, R. (2008). Ecology and genomics of Bacillus subtilis. Trends in Microbiology 16:269-275.

EFSA (2009). Guidance of the Scientific Panel of Food Contact Material, Enzymes, Flavourings and Processing Aids (CEF) on the Submission of a Dossier on Food Enzymes for Safety Evaluation.https://www.efsa.europa.eu/en/efsajournal/pub/1305

EPA (1997). *Aspergillus oryzae* – Final risk assessment. http://www.epa.gov/biotech_rule/pubs/fra/fra007.htm last visited 14 October 2014

FAO/WHO (2001). Evaluation of allergenicity of genetically modified foods. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology. http://www.who.int/foodsafety/publications/biotech/en/ec_jan2001.pdf, last visited on 12 December 2013

FAO/WHO (2006). General Specifications and Considerations for Enzyme Preparations Used in Food Processing. Compendium of food additive specifications, sixty-seventh meeting. FAO JECFA Monographs 3, 2006 (ISBN 92-5-105559-9), 63-67. http://www.fao.org/docrep/pdf/009/a0675e/a0675e00.pdf , last visited on 12 December 2013

Goodman RE, Vieths S, Sampson HA, Hill D, Ebisawa M, Taylor SL and van Ree R (2008). Allergenicity assessment of genetically modified crops--what makes sense? Nat. Biotechnol., 26 (1), 73-8

Hansen SC (1966). Acceptable daily intake of food additives and ceiling on levels of use. Food Cosmet Toxicol., 4, 427–432

Kikuchi Y, Takai T, Kuhara T, Ota M, Kato T, Hatanaka H, Ichikawa S, Tokura T, Akiba H, Mitsuishi K, Ikeda S, Okumura K and Ogawa H (2006). Crucial commitment of proteolytic activity of a purified recombinant major house dust mite allergen Der p 1 to sensitization towards IgE and IgG responses. J Immunol., 177, 1609-1617

Klein, D. A. and Paschke, M. W. (2004). Filamentous fungi: The indeterminate lifestyle and microbial ecology. Microbial Ecology 47:224-235.

Kurtzman, C.P., M.J. Smiley, C.J. Robnett, and D.T. Wicklow. 1986. DNA relatedness among wild and domesticated species of the *Aspergillus flavus* group. Mycologia 78(6):955-959.

Moshfegh AJ, Friday JE, Goldman JP, Ahuja JK. 1999. Presence of inulin and oligofructose in the diets of Americans. J Nutr. Jul;129(7 Suppl):1407S-11S.

Nakazawa T, Takai T, Hatanaka H, Mizuuchi E, Nagamune T, Okumura K and Ogawa H (2005). Multiple-mutation at a potential ligand-binding region decreased allergenicity of a mite allergen Der f 2 without disrupting global structure. FEBS Lett., 579, 1988–1994

NIH (1994). Guidelines for Research Involving Recombinant DNA Molecules. Available via https://osp.od.nih.gov/biotechnology/nih-guidelines/, accessed 02.09.2018

Nojiro K; (1984) Koji – The key product in Japanese alcoholic beverages and fermented foods. International Symposium on Agricultural Product Processing and Technology. IPB (Bogor Agricultural University

OECD (1992). Safety Considerations for Biotechnology 1992. Organisation for Economic Cooperation and Development. Part one. Elaboration of Criteria and Principles for Good Industrial Large-Scale Practice (GILSP). Available at http://www.oecd.org/env/test-no-404-acute-dermal-irritation-corrosion-9789264242678-en.htm, accessed 02 September 2018

OECD (1997a) Guidelines for Testing of Chemicals. No 471: Bacterial Reverse Mutation Assay, adopted 21 July 1997. http://www.oecd-ilibrary.org/docserver/download/9747101e.pdf?expires=1388407864&id=id&accname=guest&checksum=1B3E23FA2B678DB36257C631A956BD9E, last visited on 30 December 2013

OECD (1997c) Guidelines for Testing of Chemicals. No 474: Mammalian Erythrocyte Micronucleus Test, adopted 21 July 1997. http://www.oecd-ilibrary.org/docserver/download/9747401e.pdf?expires=1388411103&id=id&accname=guest&checksum=0311A1ADB57ADF6007EDA2A6C525E3A3, last visited on 30 December 2013

OECD (1998) Guidelines for Testing of Chemicals. No 408: Repeated Dose 90-day Oral Toxicity Study in Rodents, adopted 21 September 1998. http://www.oecd-ilibrary.org/docserver/download/9740801e.pdf?expires=1388414383&id=id&accname=guest&checksum=EE6E4D5B073A0AD147731731E6E277E5, last visited on 30 December 2013

OECD (2002) Guidelines for Testing of Chemicals. No 404: Acute Dermal Irritation / Corrosion, adopted 24 April 2002. http://www.oecd-ilibrary.org/docserver/download/9740401e.pdf?expires=1388479952&id=id&accname=guest&checksum=8F9BC27DEA84A3A7AFC37B661FEB572A , last visited on 31 December 2013

OECD (2009) Guidelines for Testing of Chemicals. No 403: Acute Inhalation Toxicity, adopted 08 September 2009. http://www.oecd-ilibrary.org/docserver/download/9740301e.pdf?expires=1388479435&id=id&accname=guest&checksum=A9B1CF2C2D49A5F0C8061417AC21AAB3 , last visited on 31 December 2013

OECD (2012) Guidelines for Testing of Chemicals. No 405: Acute Eye Irritation / Corrosion, adopted 02 October 2012. http://www.oecd-ilibrary.org/docserver/download/9712201e.pdf?expires=1388480326&id=id&accname=guest&checksum=CE6CD099AB591168A5AD79416D82094D , last visited on 31 December 2013

Parekh, S., Vinci, V. A. and Strobel, R. J. (2000). Improvement of microbial strains and fermentation processes. Applied Microbiology and Biotechnology 54:287-301.

Sanchez, S. and Demain, A. L. (2002). Metabolic regulation of fermentation processes. Enzyme and Microbial Technology 31:895-906.

Stephen AM, Champ MM, Cloran SJ, Fleith M, van Lieshout L, Mejborn H, Burley VJ. 2017. Dietary fibre in Europe: current state of knowledge on definitions, sources, recommendations, intakes and relationships to health. Nutr Res Rev. 30(2):149-190.

Takai T, Yokota T, Yasue M, Nishiyama C, Yuuki T, Mori A, Okudaira H and Okumura Y (1997). Engineering of the major house dust mite allergen Der f2 for allergen-specific immunotherapy. Nat. Biotechnol., 15, 754–758

Takai T, Ichikawa S, Yokota T, Hatanaka H, Inagaki F and Okumura Y (2000). Unlocking the allergenic structure of the major house dust mite allergen Der f 2 by elimination of key intramolecular interactions. FEBS Lett., 484, 102–107

van Loo J, Coussement P, de Leenheer L, Hoebregs H, Smits G. 1995. On the presence of inulin and oligofructose as natural ingredients in the western diet. Crit Rev Food Sci Nutr. Nov;35(6):525-52.

Valenta R (2002). The future of antigen-specific immunotherapy of allergy. Nat. Rev. Immunol., 2, 446–453

Valenta R and Kraft D (2002). From allergen structure to new forms of allergen specific immunotherapy. Curr. Opin. Immunol., 14, 718–727

Wüthrich B (1996). Enzyme als ingestive Allergene. Allergologie für die Praxis, 4, 74-91

Zittan L. (1981). Enzymatic Hydrolysis of Inulin - An Alternative Way to Fructose Production. Starch – Stärke Volume 33, Issue 11, pages 373–377.

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APPLICATION – CHECKLIST

SEPTEMBER 2018

TO:
FOOD STANDARDS AUSTRALIA NEW ZEALAND (FSANZ)

IN RELATION TO:
APPLICATION FOR APPROVAL OF ENDO-INULINASE FROM A GENETICALLY MODIFIED STRAIN OF *ASPERGILLUS ORYZAE* AS A PROCESSING AID

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CHECKLIST

General requirements (3.1)		
Check	**Page No.**	**Mandatory requirements**
☑	N/A	3.1.1 Form of application ☑ *Application in English* ☑ *Executive Summary (separated from main application electronically and in hard copy)* ☑ *Relevant sections of Part 3 clearly identified* ☑ *Pages sequentially numbered* ☑ *Electronic copy (searchable)* ☑ *All references provided*
☑	7	B Applicant details
☑	9	C Purpose of the application
☑	9 11 12	D Justification for the application ☑ *Regulatory impact information* ☑ *Impact on international trade*
☑	12	E Information to support the application ☑ *Data requirements*
☑	8	F Assessment procedure ☑ *General* ☐ *Major* ☐ *Minor* ☐ *High level health claim variation*

General requirements (3.1)		
Check	**Page No.**	**Mandatory requirements**
☑	N/A	G Confidential commercial information *Confidential material separated from other application material* *Formal request including reasons* *Non-confidential summary provided* *Note – non CCI summary is provided within the main document in the relevant sections:* *6.1.3* *6.1.4* *6.4.1*
☑	N/A	H Other confidential information ☑ *Confidential material separated from other application material* ☑ *Formal request including reasons*
☑	N/A	I Exclusive Capturable Commercial Benefit ☐ *Justification provided*
☑	13	J International and other national standards ☑ *International standards* ☑ *Other national standards*
☑	N/A	K Statutory Declaration Provided separately
☑	N/A	L Checklist/s provided with application ☑ *3.1 Checklist* ☑ *All page number references from application included* ☑ *Any other relevant checklists - Part 3.3.2*

Processing Aids (3.3.2)		
Check	**Page No.**	**Mandatory requirements**
☑	18	A.1 Type of processing aid
☑	20	A.2 Identification information
☑	21	A.3 Chemical and physical properties
☑	28	A.4 Manufacturing process
☑	32	A.5 Specification information
☑	32	A.6 Analytical method for detection
N/A	-	B.1 Industrial use information (chemical only)
N/A	-	B.2 Information on use in other countries (chemical only)
N/A	-	B.3 Toxicokinetics and metabolism information (chemical only)
N/A	-	B.4 Toxicity information (chemical only)
N/A	-	B.5 Safety assessments from international agencies (chemical only)
☑	32	C.1 Information on enzyme use on other countries (enzyme only)
☑	33	C.2 Toxicity information of enzyme (enzyme only)
☑	39	C.3. Allergenicity information of enzyme (enzyme only)
☑	41	C.4. Overseas safety Assessment Reports
☑	42	D.1 Information on source organism (enzyme from microorganism only)
☑	43	D.2 Pathogenicity and toxicity of source microorganism (enzyme from microorganism only)
☑	43	D.3 Genetic stability of source organism (enzyme from microorganism only)
☑	44	E.1 Nature of genetic modification of source organism (enzyme from GM source microorganism)
☑	52	F.1 List of foods likely to contain the processing aid
☑	54	F.2 Anticipated residue levels in foods
☑	54	F.3 Information on likely level of consumption

☑	54	F.4 Percentage of food group to use processing aid
☑	54	F.5 Information on residues in foods in other countries (if available)
☑	54	F.6 Where consumption has changed, information on likely consumption

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APPLICATION – EXECUTIVE SUMMARY

SEPTEMBER 2018

TO:
FOOD STANDARDS AUSTRALIA NEW ZEALAND (FSANZ)

IN RELATION TO:
APPLICATION FOR APPROVAL OF ENDO-INULINASE FROM A GENETICALLY MODIFIED STRAIN OF *ASPERGILLUS ORYZAE* AS A PROCESSING AID

EXECUTIVE SUMMARY

(As per section 3.1.1 A.2 of the Application Handbook 1 March 2016)

Purpose

Puratos is making this application to amend Schedule 18 – Processing Aids, of the Australia New Zealand Food Standards Code (hereafter the Code) to include the food enzyme endo-inulinase (EC 3.2.1.7) cloned in *Aspergillus oryzae* MUCL 44346 in S18-9(3) Permitted Enzymes.

The food enzyme shows inulinase activity as defined under IUBMB No EC 3.2.1.7 and is used as a processing aid in the production of fructo-oligosaccharides (FOS).

Approval is required of the enzyme as a processing aid as it is not currently approved for use in Australia and New Zealand.

Endo-inulinase does not perform any technological function in the final foods containing ingredients prepared with this enzyme. Moreover, the food products prepared with endo-inulinase do not have characteristics or nutritional value other than what is expected by the consumer.

Uses of the Food Enzyme in Food Production

Like any other enzyme, endo-inulinase acts as a biocatalyst - with the help of the enzyme, a certain substrate is converted into a certain reaction product.

The **function** of endo-inulinase is to catalyse the endohydrolysis of (2→1)-β-D-fructosidic linkages in inulin.

In general, the technological need for the enzymatic conversion of inulin with the help of endo-inulinase can be described as the production of FOS as a sugar alternative for sucrose.

The **substrate** for endo-inulinase is inulin. Inulin is polysaccharides composed of fructose unit chains of various length terminated by a single glucose unit, which can be found in Jerusalem artichoke and chicory (*Zittan 1981*). Consequently, the substrate for endo-inulinase occurs naturally in foods.

The **reaction product** of the hydrolysis of inulin with the help of endo-inulinase is a syrup of FOS.

Endo-inulinase performs its technological function during FOS production. The enzyme does not perform any technological function in the final food.

Production Method

The food enzyme object of this dossier is produced by fermentation of the microorganism *Aspergillus oryzae* in pure culture. No foreign microorganisms are allowed to develop during the enzyme manufacturing process.

Aspergillus oryzae has been used for decades for the production of food enzymes.

During the fermentation, run in closed vessels, the microorganism is provided with nutrients, water and aeration. It develops and produces the food enzyme.

After the fermentation is over, the microorganism is eliminated from the liquid broth containing the food enzyme. This broth is partially purified and concentrated, to maximize the enzyme contents.

The concentrate is then mixed with other ingredients, in order to stabilize it during storage, transportation and facilitate its use in food processing after standardisation of the commercial preparations.

The food enzyme preparation complies with international specifications (JECFA), ensuring absence of contamination by toxic substances or noxious microorganisms.

The enzyme is manufactured according to good manufacturing practices (GMP) and the principals of HACCP. When manufactured in the EU, it is also subject to Regulation (EC) No 852/2004 -Food Hygiene Regulation.

A HACCP plan is applied to the production of the enzyme to manage all potential risks that may come from fermentation**.**

Existing Authorizations of the Food Enzyme

The food enzyme endo-inulinase has been evaluated and/or authorized in the USA. Moreover, the food enzyme endo-inulinase has been submitted for evaluation to the JECFA and has been legally produced and used in the EU, where it has also been submitted for evaluation to the EFSA.

Toxicological Studies

The food enzyme object of the present application was subjected to several toxicological studies to confirm its safety for consumers. The mutagenicity studies supported 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 many times higher than those which are consumed via food.

Conclusions on the Safety of the Food Enzyme

Based on the safety of the production microorganism, on the toxicological studies, and on previous evaluations by official experts, it is concluded that the food enzyme object of this application is safe for its intended uses.

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APPLICATION – STATUTORY DECLARATION

SEPTEMBER 2018

TO:
FOOD STANDARDS AUSTRALIA NEW ZEALAND (FSANZ)

IN RELATION TO:
APPLICATION FOR APPROVAL OF ENDO-INULINASE FROM A GENETICALLY MODIFIED STRAIN OF *ASPERGILLUS ORYZAE* AS A PROCESSING AID

STATUTORY DECLARATION

(As per section 3.1.1 K of the Application Handbook as at 1 March 2016)

I, of Puratos NV (Puratos), Industrialaan 25, B-1702 Groot-Bijgaarden, Belgium; Group Public and Environmental Affairs Director make the following declaration under the *Statutory Declarations Act 1959*:

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

I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence under section 11 of the *Statutory Declarations Act 1959*, and I believe that the statements in this declaration are true in every particular.

Declared at Groot-Bijgaarden (Belgium) on 24 of September 2018

[Signature of person before whom the declaration is made]

Name:

Qualifications:

Address:

To: Food Standards Australia New Zealand

In relation to: Application for approval of endo-inulinase from *Aspergillus oryzae* as a processing aid