FOOD STANDARDS
Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

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Supporting document 1

Food technology and safety assessment – Application A1153

Endo-1,4- β-xylanase from *Trichoderma reesei* as a processing aid (enzyme)

Executive summary

FSANZ has assessed an application from AB Enzymes GmbH (AB Enzymes) to permit the use of endo-1,4-β-xylanase as a processing aid (enzyme). The enzyme is obtained from a genetically modified strain of *Trichoderma reesei (T.reesei)* expressing a xylanase gene from *Thermopolyspora flexuosa (T.flexuosa)* (previously known as *Nonomuraea flexuosa*).

Endo-1,4-β-xylanase is used for the depolymerisation of arabinoxylans in the manufacture and processing of bakery products, cereal products, grain, cereal based beverages (including beer) and potable alcohol. Use of endo-1,4-β-xylanase in these foods provides consistent quality and production efficiencies for food manufacturers.

The food technology assessment concluded that the technological purpose as a processing aid for the form and quantity are met and justified for use in bakery products, cereal products, grain, cereal based beverages (including beer) and potable alcohol. The enzyme preparation meets internationally accepted enzyme identification and accepted chemical and microbiological specifications.

The safety assessment concluded that there are no public health and safety concerns associated with the use of endo-1,4-β-xylanase from *T. reesei* as a food processing aid, based on the following considerations:

- The production organism *T. reesei* is not toxigenic or pathogenic and is absent in the final enzyme preparation proposed to be used as a food processing aid. Further, *T. reesei* has a long history of safe use as the production organism for a number of enzyme processing aids that are already permitted in the Code.
- Endo-1,4-β-xylanase from *T. reesei* was not mutagenic nor genotoxic *in vitro*.
- The no observed adverse effect level (NOAEL) in a 13-week repeat dose oral toxicity study in rats was the highest dose tested and corresponds to 1000 mg/kg bw/day or 940 mg total organic solids (TOS)/kg bw/day. This is more than 2000-fold higher than the Applicant's estimate of an individual's theoretical maximal daily intake (0.41 mg TOS/kg bw/day) based on the proposed uses, as stated in the Application.
- Endo-1,4-β-xylanase from *T. reesei* does not have the characteristics of a potential food allergen and ingestion of any residual endo-1,4-β-xylanase in food products is unlikely to pose an allergenicity concern.

From the toxicological data and in the absence of any identifiable hazard an Acceptable Daily Intake (ADI) ‘not specified’ is appropriate. A dietary exposure assessment was not required.

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1 Introduction

FSANZ received an application from AB Enzymes to amend Schedule 18 of the *Australian New Zealand Food Standards Code* (the Code) for endo-1,4 (3)-β-xylanase (EC 3.2.1.8) (endo-1,4- β-xylanase) as a processing aid. The enzyme is obtained from a genetically modified strain of *T. reesei* expressing a modified xylanase gene from *T. flexuosa (*previously known as *Nonomuraea flexuosa)*.

Arabinoxylans are the main component of hemicellulose found in plant cell walls. Addition of the endo-1,4- β-xylanase enzyme to plant based foods catalyses the hydrolysis of xylosidic linkages in arabinoxylans (and other β-1,4-linked xylans). This results in the depolymerisation of arabinoxylans into smaller oligosaccharides.

The main benefits to food manufacturers include improvements to functional properties explained in section 2.6 and ultimately consistent product quality and production efficiencies. The endo-1,4- β-xylanase enzyme is intended for use at levels of good manufacturing practice (GMP) in the manufacture and/or processing of bakery products, cereal products, grain, cereal based beverages (including beer) and potable alcohol. AB Enzymes have also provided recommended use levels for various food applications.

The endo-1,4- β-xylanase enzyme has been approved for use in France (ANSES 2015) and is Generally Recognized as Safe (GRAS) in the USA (FDA 2016).

2 Food technology assessment

2.1 Objectives of the food technology assessment

The objectives of this food technology assessment are to determine whether the proposed technological purpose is clearly stated and that the enzyme achieves its technological purpose in the form and quantity proposed for its use as a processing aid (enzyme).

2.2 Identity of the enzyme

Information regarding the identity of the enzyme included in the application has been verified using an appropriate enzyme nomenclature reference (IUBMB 2017).

IUBMB lists the accepted name as endo-1,4-β-xylanase. Similar permissions in the Code are consistent with the IUBMB accepted name so the draft variation to the Code for this Application will also follow this approach i.e. endo-1,4-β-xylanase. This is different to the Application which lists endo-1,4 (3)-β-xylanase as the common name.

Systematic name endo-1,4-β-xylanase

Common name Endo-1,4 (3)-β-xylanase
Other names Endo-(1,4)- β-xylan 4-xylanohydrolase;
endo-1,4-xylanase;
xylanase;
β-1,4-xylanase;
endo-1,4-xylanase;
endo- β -1,4-xylanase;
endo- β -1,4-D-xylanase;
1,4- β-xylan xylanohydrolase;
β-xylanase;
β-1,4-xylan xylanohydrolase;
endo-1,4- β-D-xylanase

IUBMB Enzyme Nomenclature & EC number 3.2.1.8

C.A.S. number 9025-57-4

Host microorganism *Trichoderma reesei*

Gene donor microorganism *Thermopolyspora flexuosa*

Reaction catalysed

Endo-1,4- β-xylanase hydrolyses the xylosidic linkages in an arabinoxylan backbone (and other β-1,4-linked xylans) resulting in depolymerisation of arabinoxylans into smaller oligosaccharides.

2.3 Product specification

There are international specifications for enzyme preparations used in food production. These have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006) and the Food Chemicals Codex (FCC) (USP, 2014). Both of these specification sources are primary sources listed in Schedule S3—2 of the Code. Enzyme preparations must meet these specifications.

The Application provides analytical results for several batches of endo-1,4- β-xylanase confirming the enzyme preparation meets international specifications for enzyme preparations and also Code requirements (Table 1).

Table 1 Comparison of analytical results for endo-1,4- β-xylanase enzyme preparation (batches) with international specifications and the Code requirements	**Analytical results for the endo-1,4- β-xylanase enzyme preparation (batch's)**			**JECFA**	**FCC**	**Code (section S3-4)**
	A	B	C			
Metals						
Lead (mg/kg)	<0.05	<0.05	<0.05	<5	<5	≤2
Arsenic (mg/kg)	<0.5	<0.5	<0.5			≤1
Mercury (mg/kg)	<0.1	<0.1	<0.1			≤1
Cadmium (mg/kg)	<0.05	<0.05	<0.05			≤1
Microbiology						
Coliforms (cfu/g)	<10	<10	<10	<30	<30	
E. coli (in 25 g)	ND	ND	ND	Absent		
Salmonella (in 25 g)	ND	ND	ND	Absent	Negative	
Antibiotic activity	ND	ND	ND	Absent		

† ND: not detected

2.4 Technological purpose

The technological purpose of adding endo-1,4-β-xylanase as a processing aid is to catalyse the hydrolysis of xylosidic linkages in the arabinoxylan backbone (and other β-1,4-linked xylans) found in plant cell walls resulting in depolymerisation of the arabinoxylan into smaller oligosaccharides. This results in improvements and consistency in product quality and production processes explained in section 2.6.

The optimum pH range and temperature is 6.1 to 6.5 and 80˚C, respectively. Like most enzymes, endo-1,4-β-xylanase provides a technological purpose during food processing and/or manufacturing and not in the final food, so it is categorised as a processing aid and not as a food additive.

2.5 Use levels

Food enzymes are used by manufacturers according to the *Quantum Satis* principle (consistent with GMP). This means the enzyme is used in food at the lowest level possible to provide a technological purpose. Food manufacturers adjust the use levels depending on the food application and the enzyme supplier's recommended use levels (Table 2).

Table 2 *Recommended use levels for endo-1,4-β-xylanase enzyme preparations*

Applications	**Raw material (RM)**	**Recommended use level (mg Total Organic Solids/kg raw material)**	**Final food (FF)**
Alcoholic beverages	Cereals	5	Beer
Miscellaneous	Cereals	10	Starch, fibres, gluten used in – soft drinks and beverages
Baking and other cereal products	Flour	10	Bread, baked products
Grain processing	Cereals	10	Starch, fibres, gluten

In the application, there are two forms of enzyme preparation; a liquid and powder. The approximate composition of these forms is provided in Table 3.

Table 3 *Composition of endo-1,4-β-xylanase enzyme preparations*

	Liquid form (approx. %)	**Powder form (approx. %)**
Endo-1,4-β-xylanase concentrate	23	4
Sorbitol	45	N/A
Tri-sodium, citrate dehydrate	1.5	N/A
Citric acid, anhydrous	1	N/A
Sodium benzoate	0.5	N/A
Water	29	N/A
Sunflower oil	N/A	0.5
Wheat flour	N/A	95.5

2.6 Technological justification

Endo-1,4-β-xylanase is intended for use in bakery products, cereal products, grain, beer and cereal based beverages and alcohol. The use of endo-1,4-β-xylanase results in hydrolysis of the insoluble arabinoxylans and depolymerisation to more soluble lower molecular weight arabinoxylans. Further details are provided below on the technological purpose and benefits for food applications.

2.6.1 Bakery and cereal based products

Endo-1,4-β-xylanase can be used in the manufacture of bakery products including breads, biscuits, steamed bread, cakes, pancakes, tortillas, wafers and waffles. During bakery processes endo-1,4-β-xylanase interacts with gluten and binds water by hydrolysis of arabinoxylans. This provides the following benefits:

- easier dough handling – improved extensibility and stability, reduced stickiness leading to reduced dough loss, reduced drying time for noodles and snacks
- dough structure – uniform and increased volume, improved crumb structure
- reduced batter viscosity – beneficial for making waffles, pancakes, biscuits, and
- increased firmness and reduced oil absorption in instant noodles.

2.6.2 Grain

Endo-1,4-β-xylanase helps facilitate separation of cereal components and ensure high quality of the polysaccharide and gluten fractions. The enzyme is typically added during initial steps of grain processing such as conditioning, homogenisation and dough preparation. The resulting products include flour and cereal fractions such as starch, gluten and fibre. Endo-1,4-β-xylanase is not necessarily inactivated during grain processing, but the flour and cereal fractions are used in further food processes where the enzyme will be inactivated.
The benefits for grain processing include:

- reduced viscosity of the wheat flour batter – easier gluten and starch separation
- improved gluten and starch purity, and
- breaking down arabinoxylans so milling and peeling processes are more effective.

2.6.3 Cereal based beverages including beer

In brewing, xylans present in the cell walls of the grain contribute to wort and beer viscosity – this can reduce wort and beer filtration. The addition of endo-1,4-β-xylanase helps arabinoxylan conversion resulting in reduced wort viscosity, more efficient filtration, improved extraction and reduced use of filtration aids.

2.6.4 Potable alcohol

In potable alcohol production, high levels of xylans, cellulose, lichenin and β-D-glucans result in high viscosity due to water binding capacity. High viscosity has negative effects on alcohol production as it limits solid concentration in mashing and reduces efficiency in mixing, separation and filtration processes. Endo-1,4-β-xylanase can be used before liquefaction of highly concentrated mashes to provide the following benefits:

- decreased viscosity of grain mashes
- better solid/liquid separation resulting in higher solid concentration during mashing increase in fermentable sugars
- flexibility to use more grain and less water, and
- reduced fuel consumption based on better heat transfer.

2.7 Production of the enzyme

All production processes for the endo-1,4-β-xylanase enzyme preparation are supported by quality control systems. Endo-1,4-β-xylanase is manufactured in accordance with good manufacturing practices for food and the Hazard Analysis of Critical Control Points (HACCP). The Application includes ISO 9001 and 22000 certification for production and manufacture of enzymes and HACCP certification for food safety management systems. The production site is also inspected and certified in accordance with national and European food legislation for food production.

Production includes a fermentation process, recovery process and formulation of the final commercial enzyme preparation (see Figure 1). These are summarised below with full details provided in the Application.

Part 1 - Fermentation	Inoculation Seed fermentation Main fermentation ⬇
Part 2 – Recovery	Pre-treatment Primary solid/liquid separation Concentration Polish and germ filtration ⬇
Part 3 - Final product	Formulation of enzyme preparation Packaging

Figure 1: *Manufacturing process for endo-1,4-β-xylanase*

2.7.1 Part 1 Fermentation

The enzyme is produced by a controlled submerged fermentation of a genetically modified strain of *T. reesei*. This process is commonly used for the production of food-grade enzymes.

The fermentation process involves three steps, the initial inoculum ferments to produce enough of the microorganism to begin the seed fermentation followed by the main fermentation.

2.7.2 Part 2 Recovery

The main purpose of recovery is to separate the fermentation broth into biomass and fermentation medium containing the desired enzyme protein. The desired enzyme protein is also concentrated and the ratio of enzyme activity to total organic solids (TOS) is improved.

Recovery consists of removal of the production strain and other solids, ultrafiltration and/or evaporation to concentrate the enzyme, stabilisation and then a final filtration.

2.7.3 Part 3 Final product

Food ingredients and food additives are used to formulate the enzyme preparation. The endo-1,4-β-xylanase comes in the form of a solid and liquid enzyme preparation (See Table 3). All the raw materials used in the production of the enzyme preparation are permitted food additives and processing aids in the Code (as detailed in the Application) and are appropriate for their technological purpose.

The enzyme preparation is packed into suitable food packaging before storage and distribution.

2.8 Food technology conclusion

This enzyme provides consistency and production efficiency in manufacturing and/or processing of plant based foods via hydrolysis of arabinoxylans to smaller more soluble oligosaccharides. FSANZ concluded the use of endo-1,4-β-xylanase is technologically-justified in the form and quantity proposed for use as a processing aid in the manufacture and processing of bakery products, cereal products, grain, beer and cereal based beverages and alcohol.

3 Safety assessment

3.1 Objectives for safety assessment

The objectives of this safety assessment for endo-1,4-β-xylanase are to evaluate any potential public health and safety concerns that may arise from the use of this enzyme protein, produced by a genetically modified organism, as a processing aid. Specifically by considering the:

- history of use of the host and gene donor organisms,
- characterisation of the genetic modification(s), and
- safety of the enzyme protein.

3.2 History of use

3.2.1 Host organism

T. reesei is a hypercellulolytic fungus commonly found in soil. The initial isolate came from deteriorating clothing and tent material found in the Solomon Islands after World War II. The initial isolate QM6a has been registered with the American Type Culture Collection (ATCC 13631) and has been classed as a Biosafety Level 1 organism, based on the United States Public Health Service Guidelines[1], and is not considered pathogenic to humans.

Due to the secretion of a range of cellulolytic enzymes, this fungus has been used since the 1980s for the industrial production of cellulase, hemicellulase, beta-glucanase, pectinase and xylanase for a range of industries including food (Nevalainen and Peterson, 2014; Paloheimo et al, 2016; FSANZ 2016). There is therefore a long history of safe use of *T. reesei* for the production of food grade enzymes.

[1] https://www.cdc.gov/biosafety/publications/bmbl5/index.htm

3.2.2 Gene donor organism(s)

T. flexuosa

The gene sequence for the xylanase enzyme was originally isolated from *T. flexuosa* DSM43186 (FDA, 2016). This bacterium is a gram positive actinomycete commonly found in soil. As the gene sequence has been manipulated through standard DNA cloning methods the original isolation from the donor organism, extraneous genetic material from *T. flexuosa* would not be carried across to the enzyme production organism.

Aspergillus nidulans

The gene sequence for acetamidase, used as the selection marker for positive transformants, was initially isolated from *Aspergillus nidulans* (Kelly and Hynes, 1985; Pentilla et al, 1987). The majority of *A. nidulans* strains have been classed at the Biosafety Level 1, based on the United States Public Health Service Guidelines[2], however some strains have been associated with opportunistic infections in immunocompromised individuals (Gabrielli et al, 2014; Sadarangani et al, 2015). As the gene sequence has been manipulated through standard DNA cloning methods subsequent to the original isolation from the donor organism, extraneous material from *A. nidulans* would not be carried across to the enzyme production organism.

3.3 Characterisation of the genetic modification(s)

A *T. reesei* strain was transformed using standard molecular biology methods. The transferred DNA included the endo-1,4-β-xylanase gene, a signal and carrier peptide sequence and the acetamidase gene (*amdS)* from *A. nidulans.* The latter gene allows for selection of transformants on acetamide-containing media and is not an antibiotic resistance gene.

Characterisation of inserted DNA involved Southern blotting of genomic DNA extracted from the production strain. The results showed that several copies of the insert have been integrated into the host's genome.

Genetic stability of the inserted DNA over the fermentation run was examined using Southern blot analyses of genomic DNA extracted from the production strain. The inserted DNA was confirmed to be stable.

Full details of the genetic modification to the production organism were provided as "Confidential Commercial Information".

3.4 Safety of endo-1,4-β-xylanase

In considering the safety of novel proteins it is important to consider that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects. Only a small number of dietary proteins have the potential to cause adverse health effects, because they have anti-nutrient properties or they can cause allergies in some consumers (Delaney et al. 2008). Furthermore, proteins perform a wide range of functions in humans. To encompass this range of type and function, the safety assessment of the novel protein must consider if there is a history of safe use, are there any potential toxic, anti-nutrient or allergenic effects and is the protein susceptibility to digestion.

FSANZ has previously assessed the safety of endo-1,4-β-xylanase from a range of microorganisms (NFA 1993; ANZFA 1997a, 1997b; FSANZ 2015, 2017), permissible under Standard 1.3.3. Schedule 18 lists the various permissions, including some that predate the

establishment of the National Food Authority in 1991, thereby indicating that this enzyme has a long history of safe use as a processing aid in the food industry.

The United States Food & Drug Administration (FDA) has granted Generally Recognised as Safe (GRAS) status to the enzyme preparation under the intended conditions of use (FDA, 2016). The French Agency for Food, Environment and Occupational Health & Safety (ANSES) has also approved the enzyme for the intended use (ANSES, 2015).

3.4.1 Bioinformatic analysis for potential allergenicity

The Applicant has provided the results of an *in silico* analysis comparing the endo-1,4-β-xylanase amino acid sequence inserted into *T. reesei* to known allergenic proteins listed in a range of allergen databases (Table 4).

Table 4 *List of allergen databases included in searches by the Applicant*

Allergen Database	URL
Allergen Database for Food Safety	http://allergen.nihs.go.jp/ADFS/
Allergen Online (FARRP)	http://www.allergenonline.com/
Structural Database of Allergen Proteins	http://fermi.utmb.edu/
AllerMatch	http://www.allermatch.org/

Two types of analyses were performed for this comparison:

a) 80-mer sliding window search – a FASTA alignment was performed comparing all contiguous 80 amino acids within the endo-1,4-β-xylanase sequence to the database entries. Matches were identified if there was greater than 35% homology (BLOSUM50). (Pearson and Lipman, 1988).

b) 6-mer exact match search – A FASTA alignment was performed comparing contiguous 6 amino acids within the endo-1,4-β-xylanase sequence to the database entries (FAO, 2001). Matches were identified if there was 100% homology.

No homology was found between the endo-1,4-β-xylanase protein sequence and any known allergenic proteins.

A potential allergen in the fermentation medium is wheat derived product, however the applicant has indicated that this material will be consumed in the fermentation process, and will be absent in the final product. However, the powder form of the endo-1,4-β-xylanase enzyme preparation includes wheat as an ingredient.

3.4.2 Bioinformatic analysis for potential toxicity

The Applicant provided results from *in silico* analyses comparing the amino acid sequence for the modified endo-1,4-β-xylanase protein to known protein toxins identified in the NCBI protein database. No homology was found between the modified endo-1,4-β-xylanase protein and known toxins.

3.4.3 Susceptibility of endo-1,4-β-xylanase to digestion

To confirm the digestibility of endo-1,4-β-xylanase, potential cleavage sites were investigated by FSANZ using the amino acid sequence of the modified endo-1,4-β-xylanase protein and

the PeptideCutter tool[2] in the ExPASy Proteomics Site. Endo-1,4-β-xylanase has multiple cleavage sites for pepsin (22 sites at pH 1.3 and 59 sites at pH >2), trypsin (12 sites), chymotrypsin (31 high-specificity sites, 42 low-specificity sites) and endopeptidases (13 sites). This data shows that the modified endo-1,4-β-xylanase protein is as susceptible to protein digestion as the vast majority of dietary proteins.

3.4.4 Evaluation of toxicity studies of the enzyme

The toxicity studies submitted by the Applicant were all conducted with the same endo-1,4-β-xylanase preparation, test batch No "XT Mix Lims 2003-1463-1". This is an ultra-filtrated concentrate, which is the most concentrated product before its formulation into a food enzyme preparation.

Genotoxicity

Reports of two *in vitro* genotoxicity studies have been submitted by the Applicant: a bacterial reverse mutation test (Ames test) and a chromosomal aberration test in mammalian cells.

Bacterial reverse mutation assay – Charles River Laboratories study report no 24411

The study was conducted in compliance with OECD principles of Good Laboratory Practice (GLP) and in accordance with OECD Test Guideline (TG) 471, the Bacterial Reverse Mutation Test (adopted 21 July 1997). The test item was endo-1,4-β-xylanase and the vehicle was ultra-pure water.

Tester strains used in the study were *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 plus *Escherichia coli* WP2 *uvr*A. The test was conducted by the 'treat and plate' method in the presence and absence of metabolic activation (S9 mix). Based on the results of a dose range-finding test using strain TA100, six concentrations ranging from 17 – 5000 µg endo-1,4-β-xylanase /mL were used in the main test, which was performed twice using triplicate plating.

Criteria for a positive mutagenic response were a two-fold or more increase in the number of revertant colonies compared with the negative control for *S. typhimurium* strains TA1535, TA1537 and TA98 as well as for *E. coli* WP2*uvr*A. For *S. typhimurium* strain TA100, a 1.5-fold increase over controls was considered significant. Presence of a dose-response and reproducibility of the effect was also taken into account in determining a positive result.

No toxicity was observed in either the presence or absence of S9 mix and there was no precipitation of the test item. Initial marginal increases in revertant colony counts with *S. typhimurium* TA100 and *E. coli* WP2*uvr*A treated with endo-1,4-β-xylanase were not observed upon retesting, and were therefore considered artefacts. No other evidence of mutagenic activity was observed in any of the test strains in either the presence or absence of S9 mix. Mutagenic responses with the concurrent positive controls demonstrated the validity of the test system.

Endo-1,4-β-xylanase was not mutagenic under the conditions of this study.

Chromosomal aberration assay in mammalian cells – Inveresk study report no 24406

The study was conducted in compliance with OECD principles of GLP and with OECD TG 473, the *In Vitro* Chromosomal Aberration Test. The test item was endo-1,4-β-xylanase and the vehicle was Ham's F-10 medium.

[2] http://web.expasy.org/peptide_cutter/

The test material was examined for its potential to induce chromosomal aberrations in Chinese hamster ovary (CHO) cells in both the absence and presence of metabolic activation (S9 mix). Both short-term (6 hour's exposure) and continuous (22 hours) exposure assays were conducted. Cultures in the short-term assay were harvested at 24 h post treatment for all tests, while cultures in the continuous exposure assay were harvested at 24 and 48 h post treatment. Colcemid® was added to cultures approximately 2 h before cells were harvested. Short-term treatment was conducted in the presence and absence of metabolic activation, while the continuous exposure assay was conducted only in the absence of metabolic activation. The positive control substances were methyl methane-sulphonate (MMS) in the absence of S9 and cyclophosphamide in the presence of S9.

Endo-1,4-β-xylanase was tested to the maximum recommended concentration of 5000 µg/mL in both the short-term and continuous exposure tests. Toxicity was noted in cultures harvested at 48 h post treatment at doses ≥ 1250 µg/mL. For cultures harvested at 24 h post treatment, the highest dose level assessed for chromosomal aberrations was 5000 µg/mL, while the maximum dose assessed for chromosomal aberrations in cultures harvested 48 h post treatment was 2500 µg/mL.

Cultures treated with endo-1,4-β-xylanase did not show an increased incidence of structural chromosomal aberrations compared with concurrent and historical negative control cultures. Continuous exposure cultures harvested at 48 h following endo-1,4-β-xylanase treatment were also assessed for polyploidy. No increases in the incidence of numerical chromosomal aberrations were observed.

Significant increases in the incidence of cells with structural chromosomal aberrations were observed in the positive controls compared with negative controls, demonstrating the validity of the test system.

Endo-1,4-β-xylanase did not induce structural chromosomal aberrations under the conditions of this study.

Animal studies

13-week repeated dose oral toxicity study in rats – Inveresk report no 24445

The study was performed in compliance with OECD principles of Good Laboratory Practice, and following OECD TG 408, Repeated dose 90-day oral toxicity study in rodents.

Sprague-Dawley (Crl:CD®(SD IGS BR) rats (10/sex/group) were acclimatised for 13 days prior to commencement of dosing, at which time they were approximately 7 weeks old. Animals were housed singly with *ad libitum* access to food and water, and maintained under standard laboratory husbandry conditions.

Endo-1,4-β-xylanase was administered by oral gavage at doses of 0, 250, 500 and 1000 mg/kg bw/day for 13 weeks. The vehicle and negative control was sterile water. Clinical signs and body weights were recorded daily, and food and water consumption was assessed weekly. Detailed functional observations were performed weekly, with additional detailed functional observations such as grip strength, pain perception, landing foot splay and motor activity performed pre-trial and during Week 12 of treatment. Ophthalmoscopic assessments were undertaken on all animals pre-trial and on controls and high dose animals during Week 12. Urine and blood samples were collected during Week 13. After 13 weeks of treatment all surviving animals were killed and necropsied. Tissues from all control and high dose animals, as well as animals found dead or killed prematurely, were examined histologically.

There were two premature deaths during the course of the study. A female rat in the 250 mg/kg bw/day dose group was found dead on Day 74, and a male in the 500 mg/kg bw/day

dose group was killed on Day 90. Both of these animals exhibited histological lesions consistent with incorrect dosing and these deaths were not attributed to treatment with endo-1,4-β-xylanase . All other animals survived to the end of the study.

Softer than normal faeces were noted in all treated male groups, and to a lesser extent in females administered 500 or 1000 mg/kg bw/day, from the beginning of treatment to approximately Week 8. By Week 12 only isolated incidences of softer than normal faeces were observed in males administered 500 and 1000 mg/kg bw/day. Slight but not statistically significant reductions in body weight gain were observed in males treated with 500 or 1000 mg/kg bw/day and in all groups of treated females. An initial statistically significant reduction in food consumption was seen in all female treatment groups from Days 7 to 14, while males treated with 500 or 1000 mg/kg bw/day had slightly reduced food consumption throughout the treatment period, reaching statistical significance at certain periods. These findings were not considered to be of toxicological significance.

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

The no observed adverse effect level (NOAEL) for endo-1,4-β-xylanase in this study was 1000 mg/kg bw/day, the highest dose tested. This corresponds to 940 mg total organic solids (TOS)/kg bw/day, based on a TOS content of 93.8%.

3.5 Safety assessment conclusions

There are no public health and safety concerns associated with the use of endo-1,4-β-xylanase from *T. reesei* as a food processing aid, based on the following considerations:

- The production organism *T. reesei* is not toxigenic or pathogenic and is absent in the final enzyme preparation proposed to be used as a food processing aid. Further, *T. reesei* has a long history of safe use as the production organism for a number of enzyme processing aids that are already permitted in the Code.
- Endo-1,4-β-xylanase from *T. reesei* was not mutagenic nor genotoxic *in vitro*.
- The NOAEL in a 13-week repeated dose oral toxicity study in rats was the highest dose tested and corresponds to 1000 mg/kg bw/day or 940 mg TOS/kg bw/day. This is more than 2000-fold higher than the Applicant's estimate of an individual's theoretical maximal daily intake (0.41 mg TOS/kg bw/day) based on the proposed uses, as stated in the Application.
- Endo-1,4-β-xylanase from *T. reesei* does not have the characteristics of a potential food allergen and ingestion of any residual endo-1,4-β-xylanase in food products is unlikely to pose an allergenicity concern.

Based on the reviewed toxicological data it is concluded that in the absence of any identifiable hazard an Acceptable Daily Intake (ADI) 'not specified' is appropriate. A dietary exposure assessment was therefore not required.

References

ANSES (2015) Application for extension of the authorization to use a xylanase from a strain of *Trichoderma longibrachiatum* carrying a mutated gene encoding a *Thermopolyspora flexuosa* xylanase (anterior synonym *Nonomuraea flexuosa*) in breweries, starch production and drinking alcohol production. Report prepared by the French Agency for Food, Environment and Occupational Health & Safety. (Accessed November 2017).

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