Supporting document 1

Risk and technical assessment – Application A1278

Beta-Fructofuranosidase from GM *Trichoderma reesei* as a processing aid

Executive summary

AB Enzymes GmbH (AB Enzymes) has applied to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of the enzyme beta-fructofuranosidase (EC 3.2.1.26) as a processing aid in the production of short-chain fructooligosaccharides (scFOS), and to produce a reduction in sugar (sucrose) levels in treated fruit and vegetable products. This enzyme is from a new genetically modified (GM) strain of *Trichoderma reesei* containing the beta-fructofuranosidase gene from *Aspergillus niger.*

The proposed use of this beta-fructofuranosidase as a processing aid is consistent with its known technological function. Beta-fructofuranosidase performs its technological purpose during the production of the nominated foods and is not performing a technological purpose in the final food. It is therefore functioning as a processing aid for the purposes of the Code.

There are relevant identity and purity specifications for the enzyme in the Code, and the applicant provided evidence that the enzyme meets these specifications.

T. reesei has a long history of safe use as a production microorganism of enzyme processing aids, including several that are already permitted in the Code. The production organism is neither pathogenic nor toxigenic. Analysis of the genetically modified production strain confirmed the presence and stability of the inserted DNA.

Bioinformatics analysis found no significant homology of the beta-fructofuranosidase enzyme itself with known toxins or food allergens. Studies with another enzyme, phytase, from a production strain within the same safe strain lineage as that used to produce beta-fructofuranosidase, found no evidence of genotoxicity *in vitro* or *in vivo* and no adverse effects in a 90-day oral toxicity study in rats. These findings confirm the safety of the beta-fructofuranosidase production strain. The no observed adverse effect level (NOAEL) in this study was 1000 mg total organic solids (TOS)/kg bw/day, the highest dose tested.

The theoretical maximum daily intake (TMDI) of the TOS from the beta-fructofuranosidase preparation was calculated to be 1.05 mg TOS/kg bw. A comparison of the NOAEL and the TMDI results in a Margin of Exposure (MOE) of approximately 1000.

Based on the reviewed data it is concluded that in the absence of any identifiable hazard, an

acceptable daily intake (ADI) 'not specified' is appropriate.

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1   Introduction

AB Enzymes GmbH (AB Enzymes) has applied to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of the enzyme beta-fructofuranosidase (EC 3.2.1.26). This enzyme is from a new genetically modified (GM) strain of *Trichoderma reesei* containing the beta-fructofuranosidase gene from *Aspergillus niger.*

The enzyme is intended to be used as a processing aid in the production of short-chain fructooligosaccharides (scFOS) and to produce a reduction in sugar (sucrose) levels in treated fruit and vegetable products. It is proposed to be used at the minimum level required to achieve the desired effect, in accordance with the principles of Good Manufacturing Practice (GMP[1]).

1.1   Objectives of the assessment

The objectives of this risk and technical assessment were to:

- determine whether the proposed purpose is a solely technological function and that the enzyme achieves its technological purpose as a processing aid in the quantity and form proposed to be used

- evaluate potential public health and safety concerns that may arise from the use of this enzyme, produced by a GM microorganism, as a processing aid by considering the:

    – safety and history of use of the gene donor and production microorganisms
    – characterisation of the genetic modification(s), and
    – safety of the enzyme.

2   Food technology assessment

2.1   Identity of the enzyme

The production microorganism of the enzyme is a GM strain of *T. reesei,* with the donor microorganism for the beta-fructofuranosidase gene being *A. niger*.

AB Enzymes provided relevant information regarding the identity of the enzyme, and this has been verified using the IUBMB[2] enzyme nomenclature (IUBMB 2023) reference database (McDonald and Tipton 2023). Details of the identity of the enzyme are provided in Table 1 below.

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[1] GMP is defined in the Standard 1.1.2—2 of the Code as follows: *with respect to the addition of substances used as food additives and substances used as processing aids to food, means the practice of:*
(a) limiting the amount of substance that is added to food to the lowest possible level necessary to accomplish its desired effect; and
(b) to the extent reasonably possible, reducing the amount of the substance or its derivatives that:
        (i) remains as a *component of the food as a result of its use in the manufacture, processing or packaging; and
        (ii) is not intended to accomplish any physical or other technical effect in the food itself;

[2] International Union of Biochemistry and Molecular Biology.

Table 1: Identity of the enzyme

Generic name	Beta-fructofuranosidase
IUBMB nomenclature	Beta-fructofuranosidase
Synonyms	invertase; saccharase; glucosucrase; beta-h-fructosidase; beta-fructosidase; invertin; sucrase; fructosylinvertase; alkaline invertase; acid invertase
Systematic name	beta-D-fructofuranoside fructohydrolase
IUBMB No.	EC 3.2.1.26
CAS No.	9001-57-4
Reaction	Hydrolysis of terminal non-reducing beta-D-fructofuranoside residues in beta-D-fructofuranosides

CAS: Chemical Abstracts Service

2.2 Manufacturing process

2.2.1 Production of the enzyme

Enzymes produced from microorganisms are typically produced by controlled fermentation followed by removal of the production microorganism, purification and concentration of the enzyme. Final standardisation with stabilisers, preservatives, carriers, diluents, and other approved food-grade additives and ingredients is carried out after the purification and concentration steps. The formulated enzymes are referred to as enzyme preparations, which, depending upon the application in food, may be a liquid, semi-liquid or dried product. Enzyme preparations may contain either one major active enzyme that catalyses a specific reaction during food processing or two or more active enzymes that catalyse different reactions (FAO/WHO 2018).

The information provided in the application confirms that the method of production of the enzyme is consistent with the above general information.

2.2.2 Specifications for identity and purity

There are international general specifications for enzyme preparations used in the production of food. These have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in its Compendium of Food Additive Specifications (FAO/WHO 2006) and in the Food Chemicals Codex (FCC 2022). These specifications are included in earlier publications of the primary sources listed in section S3—2 of Schedule 3 of the Code, and enzymes used as a processing aid need to meet either of these specifications. In addition, under JECFA, enzyme preparations must meet the specifications criteria contained in the individual monographs. In the case of beta-fructofuranosidase, there is no individual monograph.[3]

Schedule 3 of the Code also includes specifications for arsenic and heavy metals (section S3—4) if they are not already detailed within specifications in sections S3—2 or S3—3.

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[3] For the functional use 'enzyme preparation', the JECFA database can be searched for individual monographs: http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/en/

The applicant provided the results of analysis of three different batches of their beta-fructofuranosidase liquid concentrated preparation. Table 2 provides a comparison of the summary results of those analyses with international specifications established by JECFA and Food Chemicals Codex, as well as those in the Code. Based on those results, the enzyme met all relevant specifications.

Table 2   *Analysis of manufacturer's beta-fructofuranosidase liquid enzyme concentrated preparation compared to JECFA, Food Chemicals Codex, and Code specifications for enzymes*

Test parameters	Applicant results	Specifications		
		JECFA	**Food Chemicals Codex**	**The Code - section S3—4**
Lead (mg/kg)	<0.05	1	≤5	≤2
Arsenic (mg/kg)	<0.5	-	-	≤1
Cadmium (mg/kg)	<0.05	-	-	≤1
Mercury (mg/kg)	<0.05	-	-	≤1
Coliforms (cfu/g)	<30	≤30	≤30	-
***Salmonella* (in 25 g)**	ND	Absent	Negative	-
***Escherichia coli* (in 25 g)**	ND	Absent	-	-
Antimicrobial activity	ND	Absent	-	-

cfu = colony forming units
ND = not detected

The applicant provided additional data produced using Polymerase Chain Reaction (PCR) techniques as Confidential Commercial Information (CCI) support the applicant's claim that the enzyme preparation does not contain the production strain.

2.3   Technological function and justification

Under the current application, beta-fructofuranosidase is intended for use as a processing aid in the production of scFOS, and to produce a reduction in sugar (sucrose) levels in treated fruit and vegetable products. The applicant requested use of the enzyme at GMP levels.

As identified by the IUBMB (section 2.1, above), beta-fructofuranosidase catalyses the hydrolysis of terminal non-reducing beta-D-fructofuranoside. The enzyme also catalyses fructotransferase reactions.

FSANZ assessed the use of beta-fructofuranosidase as a processing aid in the production of scFOS as part of Application A1055 in 2013 (FSANZ 2013). Supporting Document 1 of the Approval Report for that application provided some very useful explanations of the technological purpose and justification for use of the enzyme. That information is not repeated here.

The current application notes that its beta-fructofuranosidase enzyme has its primary

function of hydrolysis to breakdown sucrose to fructose and glucose. It also has the secondary function of transfructosylation (catalyses the fructotransferase reaction) - transferring a fructose molecule from one sucrose molecule to another sucrose molecule, so producing a scFOS and glucose.

The enzyme is also proposed by the applicant to be used to reduce the concentration of sugar (sucrose) in various treated fruit and vegetable products. It uses the same technological function as for the production of scFOS (Cyninska-Antonik et al 2023, Gomes et al 2023). The enzyme is used to process raw fruit and vegetables (e.g. bananas, oranges, apples and carrots) that contain substantial sugar (sucrose) content by using the sucrose hydrolysis function and so reducing the natural concentrations of sucrose by producing scFOS in the treated food.

The enzyme is inactivated in treated food that undergoes further processing steps involving heating processes and would have no technological effect in these foods after they are produced. As well, additional processing steps post enzyme treatment including filtration steps ensures removal of the enzyme or inactivation. Therefore, the enzyme has the technological function during the processing of food and not in the final food, so it is appropriately categorised as a processing aid and not a food additive.

2.3.1   Allergen considerations

The applicant has advised that a wheat-based material (wheat bran) is used during fermentation and is wholly consumed during fermentation and absent in the final enzyme preparation. FSANZ considers that it is unlikely that the wheat-based material in the fermentation media will be present in the final commercial enzyme preparation. The Product Data Sheet states absence of cereals containing gluten (i.e. wheat, rye, barley, oats spelt, kamut) in the enzyme preparation.

2.4   Conclusion

FSANZ concludes that the use of this beta-fructofuranosidase as a processing aid in the production of scFOS, and to reduce sugar concentration in processed fruit and vegetable products, is consistent with its known technological function. FSANZ further concludes that the evidence presented to support its proposed use provides adequate assurance that the use of the enzyme, in the quantity and form proposed to be used (which must be consistent with GMP), is technologically justified and has been demonstrated to be effective in achieving its stated purpose.

Beta-fructofuranosidase performs its technological purpose during the production of the nominated foods, after which it is inactivated or removed, and is not performing a technological purpose in the final food. It is therefore functioning as a processing aid for the purposes of the Code.

There are relevant identity and purity specifications for the enzyme in the Code, and the applicant provided evidence that the enzyme meets these specifications.

3   Safety assessment

The objectives of this safety assessment are to evaluate any potential public health and safety concerns that may arise from the use of this enzyme, produced by this microorganism, as a processing aid.

Some information relevant to this section is CCI, so full details cannot be provided in this public report.

3.1 History of use

3.1.1 Host organism

The host organism of this enzyme beta-fructofuranosidase is *T. reesei* AR-996 which was derived from a QM6a strain. The original isolate QM6a is the environmental strain (Olempska-Beer et al., 2006). FSANZ already assessed the safety of *T. reesei* strains derived from QM6a as the host organism for a number of enzymes approved used as a processing aids in Schedule 18. The production strain *T. reesei* AR-996 has been derived from a safe strain lineage. The applicant provided the host identification letter from Westerdijk Fungal Biodiversity Institute and has been verified.

There is a long history of safe industrial use of *T. reesei* as a safe production of enzymes for food as well as feed processing and numerous other industrial applications. The data provided in the application shows that *T. reesei* AR-996 is safe to use as the production organism for beta-fructofuranosidase enzyme.

T. reesei QM6a strains are non-pathogenic, not known to possess any virulence factors associated with colonisation or disease, and do not present any human toxicity concerns (US EPA, 2012). Several review papers support the safety of *T. reesei* QM6a strains with no production of known mycotoxins or antibiotics under conditions used for enzyme production (Nevalainen et al., 1994; Nevalainen and Peterson, 2014; Gryshyna et al., 2016). *T. reesei* QM6a strains are known to produce peptaibol antibiotic paracelsin, but industry-standard submerged fermentation conditions are not linked to the production of paracelsin (US EPA, 2012).

T. reesei can be used under the lowest containment level at large scale, GILSP (Good Industrial Large Scale Practice), and listed as Risk Group 1 in the microorganism classification lists of the German Federal Institute for Occupational Safety and Health (BAuA, 2016) and meets the requirements of a Biosafety Level 1 organism based on the Biosafety in Microbiological and Biomedical Laboratories guidelines.

3.1.2 Gene donor organism

A synthetic beta-fructofuranosidase gene from *A. niger* was constructed using polymerase chain reaction (PCR). *A. niger* has a long history of safe use for industrial and food applications (Frisvad et al., 2018).

3.2 Characterisation of the genetic modification(s)

3.2.1 Description of DNA to be introduced and method of transformation

The gene that encodes the beta-fructofuranosidase was amplified using PCR from *A. niger*. Data provide by AB Enzymes and analysed by FSANZ confirmed the identity of the beta-fructofuranosidase enzyme.

The beta-fructofuranosidase gene was introduced into the genome of the host strain *T. reesei* using protoplast-mediated transformation (Penttilä et al. 1987; Karhunen et al. 1993), and was placed under the control of a *T. reesei* promoter and terminator. The *amdS* selectable marker gene from *A. nidulans* was used as a selective marker, enabling the selection of positive transformants by growth on media supplemented with acetamidase

(Hynes et al. 1983; Kelly and Hynes, 1985). The expression cassette was integrated at specific integration sites in the host's genome. The final production strain was selected based on growth on media containing acetamidase and high beta-fructofuranosidase activity.

3.2.2 Characterisation of the inserted DNA

Whole genome sequencing (WGS) data provided by AB Enzymes confirmed the presence of the inserted DNA in the production strain. The applicant also provided the results of Southern blot analysis which confirmed the absence of antibiotic resistance genes in the production strain.

3.2.3 Stability of the introduced DNA

The assessment confirmed the inserted gene is integrated into the genome of the production strain and does not have the ability to replicate autonomously. The inserted gene is therefore considered to be genetically stable.

To provide further evidence of the stability of the introduced beta-fructofuranosidase gene, the applicant provided Southern blot analysis and phenotypic data from large-scale fermentation of the production strain. These data confirmed that the beta-fructofuranosidase gene is expressed over multiple generations and is stable.

3.3 Safety of beta-fructofuranosidase

3.3.1 History of safe use

There does not appear to be a history of safe use for the specific beta-fructofuranosidase that is the subject of this application. The applicant has indicated that dossiers on the beta-fructofuranosidase enzyme have been submitted in Brazil, Canada, Denmark, the EU and USA, with plans to also make submissions in China, Indonesia, South Korea and Thailand.

Multiple beta-fructofuranosidase enzymes from other microbial sources are currently permitted as processing aids in Schedule 18 of the Code and/or in other countries.

3.3.2 Bioinformatic assessment of enzyme toxicity

The applicant performed a search for homology of the beta-fructofuranosidase enzyme amino acid sequence to known toxins in the NCBI Identical Protein Groups (IPG) database using BLAST-P. The search was performed in June 2022. No significant homologies were found.

3.3.3 Evaluation of toxicity studies

The applicant submitted toxicity studies performed with an enzyme (phytase) produced by a *T. reesei* strain (AR-700) in the same safe strain lineage as the production strain for beta-fructofuranosidase (AR-996). *T. reesei* AR-700 is derived from the same parental strain and host strain as *T. reesei* AR-996. This safe strain lineage concept is consistent with Food and Agriculture Organization/World Health Organization guidance on risk assessment of food enzymes (FAO/WHO 2020a).

The expression constructs are similar, only differing by the expression cassette for the enzyme gene of interest. These expression cassettes are similar and stably integrated into the genome of the strains without any additional growth/mutagenesis cycles thereafter. The manufacturing conditions for the two production strains are also similar, with only minor changes in pH and food-grade fermentation medium that are consistent with common

industry practice.

Based on the available information, the test item used in the toxicity studies of phytase from AR-700 is considered suitably equivalent for assessing the safety of *T. reesei* AR-996 and the beta-fructofuranosidase enzyme concentrate.

Details of the batch number and purity of the phytase test item used in the toxicity studies reviewed below are CCI.

Animal studies

90-day toxicity study in rats ([Redacted] 2011a) Regulatory status: GLP; conducted in accordance with OECD Test Guideline (TG) 408 (1998)

In a subchronic toxicity study, phytase was administered to Wistar (RccHan) rats (10/sex/group) by oral gavage at doses of 0, 100, 300 or 1000 mg TOS/kg bw/day for 91 days. Water was used as the vehicle control. Clinical signs were recorded daily, food consumption and body weights were recorded weekly. A functional observational battery and assessment of locomotor activity and grip strength were performed during week 13. Ophthalmoscopy examinations were conducted on control and high dose animals on day 88. At the end of the study blood samples were collected for haematology and clinical chemistry assessment and urine samples collected for urinalysis. Animals were killed, necropsied and examined post mortem. Histological examinations were performed on organs and tissues from all control and high dose groups, and all gross lesions from animals in the low and mid dose groups.

One male in the high dose group was found dead on day 13 of treatment. The cause of death was considered by the study authors to be possibly a dosing error, but not the result of systemic toxicity based on an absence of clinical signs or macroscopic/microscopic observations. All other animals survived to the end of the study and no treatment-related clinical signs were observed. No treatment-related differences were observed during the functional observational battery, grip strength and locomotor activity assessments. Food consumption was similar in all groups. There were no treatment-related effects on body weight in males. Body weights were increased in females in the high dose group compared to controls (+ 5.3%). This was considered to be a mild, likely pharmacodynamic effect of the test item but was not considered adverse. No treatment-related adverse effects were observed on ophthalmoscopy and haematology parameters. Sodium levels were slightly elevated in high dose males and females compared with controls. This change was considered to be test item-related but not adverse. There were no treatment-related changes in other clinical chemistry parameters, urinalysis, organ weights, macroscopic and microscopic observations.

It was concluded that the no observed adverse effect level (NOAEL) was 1000 mg TOS/kg bw/day, the highest dose tested.

Genotoxicity

Bacterial reverse mutation assay ([Redacted] 2010) Regulatory status: GLP; conducted in accordance with OECD TG 471 (1997)

Phytase was tested for its potential mutagenicity in bacterial cells using the *Salmonella enterica* ser. Typhimurium strains TA1535, TA1537, TA98 and TA100, and *Escherichia coli* strain WP2 *uvr*A. The test was initially conducted by the plate incorporation method and then repeated using the pre-incubation method. Both assays were performed in the presence and

absence of metabolic activation (S9 mix). Phytase was tested at a range of concentrations up to 5000 µg/plate. The vehicle control was water. Positive controls in the absence of metabolic activation were sodium azide, 4-nitro-o-phenylene-diamine or methyl methane sulfonate. The positive control in the presence of metabolic activation was 2-aminoanthracene.

No cytotoxicity was observed. There were no substantial increases in the number of revertant colonies compared with negative controls following treatment with phytase in either test, in the presence or absence of metabolic activation. The positive controls produced the expected increases in revertant colonies, confirming the validity of the test system.

It was concluded that phytase was not mutagenic under the conditions of this study.

In vitro chromosome aberration test ([Redacted] 2011b) Regulatory status: GLP; conducted in accordance with OECD TG 473 (1998)

The potential for phytase to induce structural chromosome aberrations *in vitro* was evaluated in Chinese hamster V79 cells. Two independent experiments were performed in duplicate using phytase concentrations up to 5000 µg/mL. In Experiment I, the exposure period was 4 hours with and without metabolic activation (S9 mix). In Experiment II the exposure period was 4 hours with S9 mix and 18 hours without S9 mix. The vehicle control was water. Positive controls were cyclophosphamide and ethylmethane sulfonate in the presence and absence of S9, respectively. Chromosomes were prepared 18 hours after start of treatment with the test item. At least 100 metaphases per culture were evaluated for structural chromosome aberrations.

No cytotoxicity was observed in either experiment. No clastogenicity was observed following treatment with phytase in the absence of S9 for either 4 or 18 hours. In the presence of S9 a statistically significant increase in the number of aberrant cells, excluding gaps, was observed at the highest concentration in Experiment I. This value was only slightly higher than the laboratory's historical solvent control range and was considered to be a result of high variability between the duplicate cultures at 5000 µg/mL: the aberration frequency only exceeded the historical control range in one of the two cultures. When the assay was repeated as part of Experiment II there was no increase in the chromosome aberration frequency compared with vehicle controls. No evidence of an increase in polyploid metaphases was observed following treatment with phytase. The positive controls produced the expected increases in frequency of chromosome aberrations, confirming the validity of the test system.

It was concluded that phytase was not clastogenic under the conditions of this study.

In vivo micronucleus assay in rats ([Redacted] 2011c) Regulatory status: GLP; conducted in accordance with OECD TG 474 (1997)

The potential for phytase to induce micronuclei in polychromatic erythrocytes (PCE) in bone marrow of rats was investigated. Following a preliminary study of acute toxicity using 2000 mg/kg bw phytase (2 rats/sex), groups of 7 male Wistar rats (age 4-5 weeks) were administered single gavage doses of 0, 500, 1000 and 2000 mg/kg bw phytase in 0.9% saline. A positive control group was administered cyclophosphamide (20 mg/kg bw). Animals were killed 24 hours following treatment and bone marrow cells collected for micronuclei analysis. An additional group was administered 2000 mg/kg bw phytase and assessed for micronucleus induction 48 hours following treatment. 2000 PCEs per animal were scored for the presence of micronuclei. The ratio between PCEs and normochromatic erythrocytes (NCEs) was also assessed.

In the preliminary study rats administered 2000 mg/kg bw showed a reduction in spontaneous activity from 1 – 24 hours following treatment and ruffled fur for up to 48 hours. In the main experiment rats in the high dose phytase group exhibited ruffled fur at 2 – 6 hours following treatment. No other clinical signs were observed in this group, and no clinical signs were observed at lower doses. No effects on the ratio of PCEs to NCEs were observed following treatment with phytase compared with the vehicle controls, indicating a lack of cytotoxicity in the bone marrow.

There were no increases in the frequency of micronucleated PCEs following treatment with phytase compared with controls. All values were within the laboratory's historical vehicle range. The positive control induced a significant increase in micronucleus frequency.

A limitation of this study is that bone marrow exposure to the test item was not confirmed given the absence of bone marrow cytotoxicity or data on toxicokinetics. However clinical signs of toxicity suggesting possible systemic exposure were observed.

It was concluded that phytase did not induce micronuclei in rat bone marrow cells under the conditions of this study.

3.3.4   Potential for allergenicity

Searches for homology of the beta-fructofuranosidase amino acid sequence with those of known allergens were performed in 2022 by the applicant using the AllergenOnline database[4]. The following searches were conducted:

- Alignment (FASTA) of the entire query amino acid sequence to known allergens (more than 35% identity)
- Alignment (FASTA) of sliding 80-amino acid windows of the query protein to known protein allergens (more than 35% identity)
- A search for 8 amino acid exact searches.

No matches of greater than 35% identity were found using the full-length search or the 80-mer sliding window search. No exact matches of 8 amino acids were found.

As the AllergenOnline database was updated in May 2023, FSANZ repeated the above searches in August 2023. No matches were found.

A search of the scientific literature found no reports of food allergy relating to the beta-fructofuranosidase enzyme from GM *T. reesei*. A beta-fructofuranosidase from tomato has been identified as a potential tomato food allergen based on IgE antibody reactivity (Sol l 2, Foetisch et al 2003). No significant homology with this allergen was identified in the above searches, however, and other recombinant, non-glycosylated forms of beta-fructofuranosidase have been shown not to trigger immune responses in sera from patients with tomato allergy (Westphal et al 2003).

Based on the available information the beta-fructofuranosidase enzyme is not expected to pose a food allergenicity concern.

3.4   Dietary exposure assessment

The objective of the dietary exposure assessment was to review the budget method calculation presented by the applicant as a 'worst-case scenario' approach to estimating likely levels of dietary exposure, assuming that all of the TOS from the β-fructofuranosidase

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[4] AllergenOnline: http://www.allergenonline.org/

preparation remained in the food.

The budget method is a valid screening tool for estimating the theoretical maximum daily intake (TMDI) of a food additive (Douglass *et al* 1997). The calculation is based on physiological food and liquid requirements, the food additive concentration in foods and beverages, and the proportion of foods and beverages that may contain the food additive. The TMDI can then be compared to an acceptable daily intake or a NOAEL to estimate a margin of exposure (MOE) for risk characterisation purposes. Whilst the budget method was originally developed for use in assessing food additives, it is also appropriate to use for estimating the TMDI for processing aids (FAO/WHO 2020b). The method is used by international regulatory bodies and the FAO/WHO Joint Expert Committee on Food Additives (JECFA) (FAO/WHO 2021) for dietary exposure assessments for processing aids.

In their budget method calculation, the applicant made the following assumptions:
- the maximum physiological requirement for solid food (including milk) is 25 g/kg bw/day
- 50% of solid food is processed
- all solid foods contain a ratio of raw material to the final food of 1 (1 kg fruit produces 1 kg fruit puree)
- the maximum physiological requirement for liquid is 100 ml/kg bw/day (the standard level used in a budget method calculation for non-milk beverages)
- 25% of non-milk beverages are processed
- all non-milk beverages contain a ratio of raw material to the final food of 1.3 (1.3 kg fruit produces 1 L fruit juice)
- all solid foods and non-milk beverages contain the highest use level of 14 mg TOS/kg in the raw material;
- all of the TOS from the enzyme preparation remains in the final food;
- all producers of fruit and vegetable juices, jams and fruit purees would use this β-fructofuranosidase preparation;
- the final foods containing the theoretical amount of the β-fructofuranosidase preparation would be consumed daily over the course of a lifetime.

Based on these assumptions, the applicant calculated the TMDI of the TOS from the enzyme preparation to be 0.63 mg TOS/kg bw/day.

As assumptions made by the applicant differ from those that FSANZ would have made in applying the budget method, FSANZ independently calculated the TMDI using the following assumptions that are conservative and reflective of a first tier in estimating dietary exposure:
- All non-milk beverages contain a ratio of raw material to the final food of 2.0 (2.0 kg fruit produces 1 L fruit juice), based on FSANZ's raw equivalence factors for fruit and vegetable juices sold in Australia and New Zealand.

- The maximum physiological requirement for solid food (including milk) is 50 g/kg bw/day (the standard level used in a budget method calculation where there is potential for the enzyme preparation to be in baby foods or general purpose foods that would be consumed by infants).

- While the default proportions noted in the FAO/WHO Environmental Health Criteria (EHC) 240 Chapter 6 on dietary exposure assessment (FAO/WHO 2020b) is that 12.5% of solid foods contain the enzyme, the applicant has assumed a higher proportion of

50% based on the nature and extent of use of the enzyme. FSANZ has therefore also used this proportion for solid foods as a worst-case scenario.

All other inputs and assumptions used by FSANZ remained as per those used by the applicant. The TMDI of the TOS from the enzyme preparation based on FSANZ's calculations for solid food and non-milk beverages is 1.05 mg TOS/kg bw/day.

The estimate of the TMDI undertaken by the applicant and FSANZ will be overestimates of the dietary exposure given the conservatisms in the budget method. This includes the assumption that all of the TOS from the enzyme preparation remains in the final foods and beverages whereas the applicant has stated that the enzyme is likely to either be inactivated or removed during processing. If any inactivated enzyme remained after processing, it would be present in insignificant quantities and perform no function in the final food.

4 Discussion

No public health and safety concerns were identified in the assessment of beta-fructofuranosidase produced by GM *T. reesei*.

T. reesei has a long history of safe use as a production microorganism of enzyme processing aids, including several that are already permitted in the Code. The production organism is neither pathogenic nor toxigenic. Analysis of the genetically modified production strain confirmed the presence and stability of the inserted DNA.

Bioinformatics analysis found no significant homology of the beta-fructofuranosidase enzyme itself with known toxins or food allergens. Studies with another enzyme, phytase, from a production strain within the same safe strain lineage as that used to produce beta-fructofuranosidase, found no evidence of genotoxicity *in vitro* or *in vivo* and no adverse effects in a 90-day oral toxicity study in rats. These findings confirm the safety of the beta-fructofuranosidase production strain. The NOAEL in this study was 1000 mg TOS/kg bw/day, the highest dose tested.

The TMDI of the TOS from the β-fructofuranosidase preparation was calculated to be 1.05 mg TOS/kg bw/day. A comparison of the NOAEL and the TMDI results in a margin of exposure (MOE) of approximately 1000.

5 Conclusion

Based on the reviewed data it is concluded that in the absence of any identifiable hazard an Acceptable Daily Intake (ADI) 'not specified' is appropriate.

6   References

[Redacted] (2010) Salmonella typhimurium and Escherichia coli reverse mutation assay.

[Redacted] (2011a) 90-day oral toxicity (gavage) study in the Wistar rat.

[Redacted] (2011b) In vitro chromosome aberration test in Chinese hamster V79 cells.

[Redacted] (2011c) Micronucleus assay in bone marrow cells of the rat.

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