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**[135–20]**

## **Supporting document 1**

Risk and technical assessment report – Application A1194

### Glucoamylase from GM *Trichoderma reesei* as a PA (enzyme)

#### **Executive summary**

The purpose of the application is to amend Schedule 18 – Processing Aids of the Australia New Zealand Food Standards Code (the Code) to include the enzyme glucoamylase (EC 3.2.1.3), from a genetically modified (GM) strain of *Trichoderma reesei*. This production organism contains the glucoamylase gene from a different strain of *Trichoderma reesei*. Glucoamylase is proposed as a processing aid for the manufacture of bakery products, brewed products, potable alcohol and in starch processing.

The evidence presented to support the proposed use of the enzyme provides adequate assurance that the enzyme, in the quantity and form proposed to be used, is technologically justified and has been demonstrated to be effective in achieving its stated purpose. The enzyme meets international purity specifications.

The safety assessment concluded that the use of the enzyme under the proposed conditions is safe. The *T. reesei* host is neither pathogenic nor toxigenic and analysis of the GM production strain, which has a history of safe use in food production, confirmed the presence and stability of the inserted DNA.

The glucoamylase enzyme that is the subject of this application has a high degree of amino acid homology to that assessed previously by JECFA in 2013. JECFA identified a NOAEL (No Observed Adverse Effect level) of 166.4 mg/kg bw/day total organic solids (TOS), the highest dose tested. The glucoamylase preparation was not genotoxic in a bacterial reverse mutation assay or a chromosomal aberration test. A closely related trehalase enzyme from host strain M1-1.1 was not genotoxic *in vitro* and caused no adverse effects in a 13-week repeat dose toxicity study in rats. The NOAEL was 1000 mg/kg bw/day TOS, the highest dose tested. The applicant's estimated theoretical maximal daily intake (TMDI) based on the proposed use pattern is 3.18 mg/kg bw/day TOS. A comparison of this value with the NOAEL of the closely-related trehalase (1000 mg/kg/day TOS) enzyme indicates that the Margin of Exposure between the NOAEL and TMDI is more than 300.

Bioinformatic analysis indicated that the enzyme has no significant homology with any known toxins or food allergens, and is unlikely to pose an allergenicity or toxicity concern. The applicant has indicated that glucose is included in the fermentation medium and the final formulation. 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.

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# 1 Introduction

Danisco New Zealand Ltd applied to FSANZ for permission to use the enzyme glucoamylase (EC 3.2.1.3) as a processing aid in the manufacture of bakery products, brewed products, potable alcohol and in starch processing. This glucoamylase is from a genetically modified (GM) strain of *Trichoderma reesei*, containing the glucoamylase gene from a different strain of *T. reesei*.

There are permissions for glucoamylase from GM and non-GM microbial sources in the Code, however, not when produced from this particular source. If permitted following a pre-market assessment, Danisco's glucoamylase will provide an additional option for manufacturers of bakery products, brewed products, potable alcohol and starch processors.

## 1.1 Objectives of the assessment

The objectives of this risk and technical assessment were to:

- determine whether the proposed purpose is clearly stated and that the enzyme achieves its technological function in the quantity and form proposed to be used as a food processing aid
- 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. Specifically by considering the:
  - history of use of the gene donor and production microorganisms
  - characterisation of the genetic modification(s), and
  - safety of the enzyme.

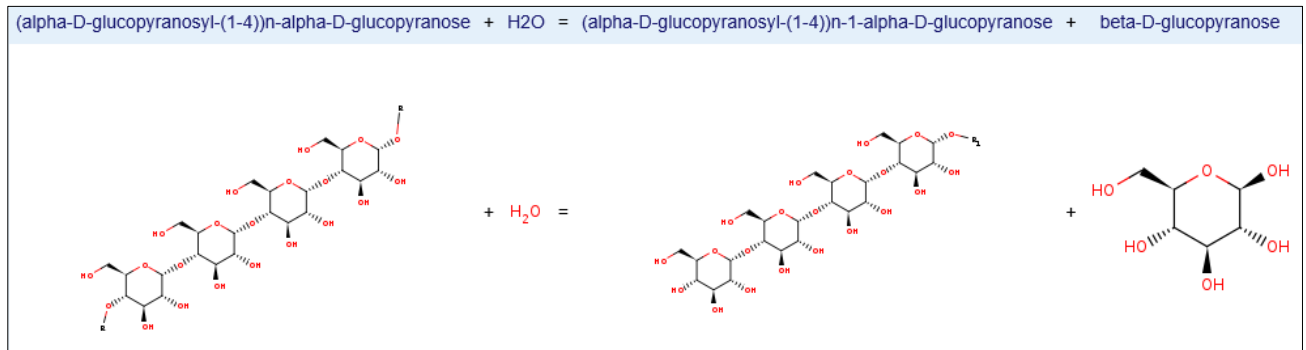
# 2 Food technology assessment

## 2.1 Characterisation of the enzyme

### 2.1.1 Identity of the enzyme

Danisco provided relevant information regarding the identity of the enzyme, and this has been verified using an appropriate enzyme nomenclature reference (IUBMB 2017).

Accepted IUBMB <sup>1</sup> name:	glucan 1,4- $\alpha$ -glucosidase <sup>2</sup>
Systematic name:	4- $\alpha$ -D-glucan glucohydrolase
Other names:	<b>glucoamylase</b> ; amyloglucosidase; $\gamma$ -amylase; lysosomal $\alpha$ -glucosidase; acid maltase; exo-1,4- $\alpha$ -glucosidase; glucan 1,4- $\alpha$ -glucosidase; glucose amylase; $\gamma$ -1,4-glucan glucohydrolase; 1,4- $\alpha$ -D-glucan glucohydrolase,
IUBMB enzyme nomenclature:	EC 3.2.1.3
CAS <sup>3</sup> number:	9032-08-0
Reaction:	Glucoamylase degrades polysaccharides such as starch, amylose, amylopectin, dextrin and glycogen by hydrolysis of 1,4- $\alpha$ linkages successively with release of $\beta$ -D-glucose (Figure 1).



Source: BRENDA:EC3.2.1.3 (<https://www.brenda-enzymes.org/enzyme.php?ecno=3.2.1.3>)

**Figure 1** Representation of hydrolysis reaction of a polysaccharide catalysed by glucoamylase

### 2.1.2 Technological purpose of the enzyme

The two-fold action of glucoamylase on large, branched starch polysaccharides is of technological importance in both starch processing and alcohol production. This is because, in addition to its action on terminal glucose units connected by 1,4- $\alpha$ -D-glucosidic bonds, it hydrolyses glucose units at branch points along the polysaccharide chain, connected in a 1,6- $\alpha$ -D-glucosidic conformation. These branch points could otherwise remain as non-

<sup>1</sup> International Union of Biochemistry and Molecular Biology.

<sup>2</sup> Although the accepted IUBMB name is 'glucan 1,4- $\alpha$ -glucosidase', the name used in the application, this document and the Call for Submissions is 'glucoamylase'. This is the name that will be used in the proposed draft variation to the Code for this enzyme.

<sup>3</sup> Chemical Abstracts Service.

fermentable dextrans, resulting in an incomplete, inefficient degradation of starch.

Glucoamylase's conversion of polysaccharides such as starch, amylose, amylopectin and dextrin benefits the processing of food raw materials naturally containing the substrate. In baking, during dough or batter process, glucoamylase reduces the need to add simple sugars to the formulation, facilitating production of 'no sugar added' yeast-fermented breads. Also, as yeast vigour may be reduced by high sugar levels, this enables lower yeast additions during the processing of baked products. Glucoamylase also reduces the crumb firming and therefore rate of staling in baked goods caused by starch retrogradation (Damodaran et al, 2008).

In brewing, glucoamylase maximises conversion of starchy substrates to fermentable carbohydrate, providing potential for higher alcohol yield and increased beer attenuation. It is used during the mashing stage of malted cereal, cereal and other plant sources including barley, maize, wheat, rye, sorghum, rice, tapioca and potatoes. Glucoamylase is denatured in the following lautering or mash filtration step, however may also be added during the fermentation step to hydrolyse liquefied starch to glucose, which is fermented to ethanol. Any glucoamylase used during fermentation is then denatured during the pasteurization step.

During potable alcohol production, glucoamylase is added in the pre-treatment, liquefaction, pre-saccharification or the fermentation step. At the end of fermentation, the solids are separated from the fermentation slurry thus removing any enzyme protein precipitate. The liquids are then distilled and subsequently filtered through a molecular sieve at temperatures well over boiling point to adsorb further traces of water and water-soluble protein.

When used in starch processing, glucoamylase saccharifies liquefied starch from various sources such as maize, wheat, milo, tapioca, barley, rice and potatoes. The resultant glucose-rich syrups can be purified to meet various specifications, crystallized to produce dextrose, isomerized to produce high fructose corn syrup, or fermented to produce organic acids such as citric acid, lactic acid, various amino acids, alcohol and other food ingredients.

The stated technological purpose of glucoamylase in starch processing and alcohol production is supported by scientific literature (Poulson 1983; Reichelt 1983; Schuster et al. 2002).

Danisco provided analysis to demonstrate that this particular glucoamylase can be used at slightly broader temperature range and pH range than typical for glucoamylase. Table 1 details this, plus a range of other physical and chemical properties of the enzyme preparation.

**Table 1** *Glucoamylase enzyme preparation physical/chemical properties*

Physical/chemical properties of commercial enzyme preparation	
Enzyme activity	>487 GAU* (Spec), actual; 554 avg from 3 batch results provided
Appearance	Liquid, tan to brown in colour
Temperature range	Activity within range 30 - 90°C Optimum 63-73°C
Temperature stability	80% @ 55°C up to 180 min.  <40% relative activity remained @ 60°C after 120 min. Below detection of activity @ 70°C for <10 min
pH range	Max activity @ 4.5  Activity within range 3.0-6.5
Storage stability	Approx. 90% remaining activity when stored for 18 months @ 4°C, approx. 85% @ ambient temp

\*GAU – One GAU is defined as the amount of glucoamylase that releases one gram of glucose per hour (= 5.6 mmol of glucose per hour) from soluble starch substrate at pH 4.3 and temperature of 30 °C

Use of commercial enzyme preparations should follow Good Manufacturing Practice (GMP), where use is at a level that is not higher than that necessary to achieve the desired enzymatic reaction. The conditions of use of the enzyme during food processing will depend on a number of factors including the nature of the application and the individual food manufacturers' production processes. The optimum use level should be assessed and adjusted using trials that reflect their particular processes, whilst providing an acceptable economic cost (Table 2).

**Table 2** *Indicative usage levels of glucoamylase*

Application	Raw Material (RM)	Recommended usage levels (mg *TOS/kg RM)	Maximum recommended levels (mg TOS/kg RM)
Baking	Flour	25-224	224
Brewing	Cereals	25-1120	1120
Potable alcohol production	Cereals	25-230	230
Starch processing	Cereals	40-115	115

\*To distinguish the proportion of the enzyme preparation derived from the source material and manufacturing process from that contributed by intentionally added formulation ingredients, the content of total organic solids (TOS) is calculated as follows: % TOS = 100 - (A + W + D) where: A = % ash, W = % water and D = % diluents and/or other formulation ingredients.

### **2.1.3 Technological justification for the enzyme**

As outlined above, the application indicates that the enzyme fulfils an important technological purpose and provides the food and beverage industry with an opportunity to improve the consistency of their products, gain processing efficiencies and any cost savings associated with this. Benefits may also include increased flexibility in the choice of and a reduction in the use of raw materials used and therefore waste reduction.

## **2.2 Manufacturing process**

### **2.2.1 Production of the enzyme**

The enzyme is produced by fermentation of the GM strain of *T. reesei* via a three-part process consisting of fermentation, recovery through separation of cell mass from enzyme followed by concentration/purification, formulation and drying, to form the stable enzyme.

The raw materials used in the fermentation and recovery process are standard ingredients used in the enzyme industry. Most raw materials conform to the specifications of the Food Chemical Codex, 6th edition (FCC 2008) and for those which do not appear in the FCC, Danisco has internal requirements in line with FCC requirements and has in place a supplier quality program. Danisco manufacture its glucoamylase in accordance with food good manufacturing practice (GMP) with the resultant product meeting the general requirements for enzyme preparations of the Food Chemicals Codex, Sixth Edition (FCC 2008) and the General Specifications for Enzyme Preparations Used in Food.

Full details on the manufacturing process, raw materials and ingredients used in the production of Danisco's glucoamylase were provided as "Confidential Commercial Information".

### **2.2.2 Allergen considerations**

Glucose syrup used during fermentation may be sourced from wheat on occasion, however if used, wheat protein will be conservatively present at only 5ppb in the final food. In the unlikely scenario that any wheat protein from glucose syrup was present in the enzyme preparation, and considering the low usage level of enzymes in a properly controlled potable alcohol distillation process, wheat proteins or peptides would not be carried over into the distillate (EFSA 2007, Cressey 2010).

### **2.2.3 Specifications**

The JECFA Compendium of Food Additive Specifications (2017) and the Food Chemicals Codex 11<sup>th</sup> edition (2018) are international specifications for enzymes used in the production of food. These are primary sources of specifications listed in section S3—2 of Schedule 3 of the Code. Enzymes need to meet these specifications. Schedule 3 of the Code also includes specifications for heavy metals (section S3—4) if they are not already detailed within specifications in sections S3—2 or S3—3.

Table 3 provides a comparison of the analysis of different batches of glucoamylase with international specifications established by JECFA and Food Chemicals Codex, as well as those in the Code (as applicable). Based on these results, the enzyme preparation meets all relevant specifications.

**Table 3** Analysis of enzyme glucoamylase compared to JECFA, Food Chemicals Codex, and Code specifications for enzymes (3 batches)

Test parameters	Danisco test results	Specifications		
		JECFA	Food Chemicals Codex	The Code (section S3—4)
Lead (mg/kg)	<0.05 – 0.1	≤ 5	≤ 5	≤2
Arsenic (mg/kg)	<0.1	-	-	≤1
Cadmium (mg/kg)	<0.01	-	-	≤1
Mercury (mg/kg)	<0.01	-	-	≤1
Coliforms (cfu/g)	<1	≤30	≤30	-
<i>Salmonella</i> (in 25 g)	Negative	Absent	Negative	-
<i>E. coli</i> (in 25 g)	Negative	Absent	-	-
Antimicrobial activity	Negative	Absent	-	-

## 2.3 Food technology conclusion

The proposed use of this glucoamylase in the manufacture of bakery products, brewed products, potable alcohol and in starch processing is clearly described in the application. FSANZ concludes that Danisco has provided adequate assurance to support the proposed use of the enzyme, when used at GMP levels and technological justification in achieving its function. Danisco has also exhibited consistency in manufacture with the enzyme meeting its own specifications and also international purity specifications.



## 3 Safety assessment

### 3.1 History of use

#### 3.1.1 Host organism

*T. reesei* is a common, hypercellulolytic, soil fungus that was initially isolated from deteriorating canvas made from cellulosic material. The original isolate QM6a is the type strain for *T. reesei* (Olempska-Beer et al., 2006) and has been registered with the American Type Culture Collection under ATCC13631. Due to the secretion of a range of cellulolytic enzymes, this fungus has been used since the 1980s for the industrial production of enzymes for a range of industries, including food (Nevalainen and Peterson, 2014; Paloheimo et al., 2016).

FSANZ has previously assessed the safety of *T. reesei* as the source organism for several enzymes used as processing aids. In this application, production strain TrGA-LOVMC2#3 was derived from *T. reesei* strain M1-1.1, which is derived from RL-P37, which is in turn derived from QM6a. The applicant has previously confirmed RL-P37 as *T. reesei* in application A1174 (FSANZ 2020). This confirmation was based on 100% DNA sequence identity (over 768 bp) between RL-P37 and the *T. reesei* type strain ATCC13631 at specific conserved DNA regions.

In humans, *T. reesei* is not pathogenic and meets the requirements of a Biosafety Level 1 organism based on the [Biosafety in Microbiological and Biomedical Laboratories guidelines](#). Although some *T. reesei* strains can produce mycotoxins, most industrial production strains do not produce mycotoxin or antibiotics under conditions used for enzyme production (Hjortkjaer et al., 1986; Nevalainen et al., 1994; Blumenthal 2004). The Applicant provided an analytical report (Nielson, 2011) demonstrating that no known mycotoxins, including Trichothecenes, were shown to be produced by the host strain M1-1.1.

#### 3.1.2 Gene donor organisms

The gene for the glucoamylase enzyme was isolated from *T. reesei* QM6a by PCR.

##### *Regulatory and other genetic elements*

The glucoamylase gene was placed under the control of promoter and terminator sequences derived from the *T. reesei* cellobiohydrolase gene *cbh1*.

The two rounds of transformation of *T. reesei* with the glucoamylase gene utilised two separate selectable markers.

The first transformation utilised the selectable marker gene for acetamidase (*amdS*) from *Aspergillus nidulans* (Kelly and Hynes, 1985). This organism meets the criteria for a Biosafety Level 1 agent not associated with disease in healthy human adults, but has been associated with infections in immunocompromised individuals (Gabrielli et al, 2014). Inclusion of the *amdS* marker gene is regarded as 'mainstream' in filamentous fungi systems involving recombinant gene expression (Gryshyna et al., 2016), and there are no safety concerns with the acetamidase protein product.

The orotate phosphoribosyl transferase (*pyr2*) gene from *T. reesei* was used as a selectable marker during the second transformation.

## 3.2 Characterisation of the genetic modification(s)

### 3.2.1 Description of DNA to be introduced and method of transformation

Two plasmids were generated to introduce the glucoamylase and selectable marker genes into the *T. reesei* host.

The first plasmid contained an expression cassette including: the glucoamylase gene under control of *T. reesei cbh1* gene regulatory sequences; and the *amdS* selectable marker gene from *A. nidulans*. The *amdS* gene allowed for selection of positive transformants by growth on media containing acetamide (Kelly and Hynes 1985).

In the second plasmid, the *amdS* gene in the expression cassette was replaced by the *T. reesei pyr2* gene. The addition of the *pyr2* gene restored the aspartate carbamoyltransferase enzyme function, allowing for selection of positive transformants of a *pyr2<sup>-</sup>* strain by growth on minimal media devoid of uridine (Jørgensen et al., 2014).

The *amdS*-containing expression cassette was excised from its plasmid by restriction enzyme digestion; purified by agarose gel electrophoresis; and transformed by PEG-mediated protoplast fusion into a *T. reesei* strain derived from parental strain RL-P37.

This intermediate strain was subjected to several rounds of chemical and directed mutagenesis; after which, the integrity of the inserted copies of the glucoamylase gene was confirmed by sequencing. One suitable glucoamylase-expressing *pyr2<sup>-</sup>* mutant strain derived from the first transformation was then transformed with the PCR-amplified *pyr2*-containing expression cassette. A spore-purified transformant was designated as the production strain TrGA-LOVMC2#3.

### 3.2.2 Characterisation of the inserted DNA

Southern blot analysis using a probe targeting the glucoamylase gene sequence was performed on genomic DNA extracted from the production strain, its parental strain and suitable control strains. Analysis of DNA digested with two individual restriction enzymes showed that multiple copies of the glucoamylase-*pyr2* and glucoamylase-*amdS* expression cassettes were integrated into the genome of the TrGA-LOVMC2#3 production strain. The data was insufficient to quantify the number of cassettes integrated in the production strain.

Hybridisation with a probe targeting the backbone of the vector from which the expression cassette was cleaved and purified confirmed its absence from the production strain's genome—including the antibiotic resistance marker genes used when passaging the expression vectors in *E. coli*.

### 3.2.3 Stability of the production organisms and inheritance of the introduced DNA

The stability of the introduced DNA in the production strain was demonstrated by Southern blot analysis of restriction enzyme digested genomic DNA samples extracted after the end of three separate large-scale commercial fermentation runs, using DNA from a 24 hour shake flask stock culture as control. The blot was probed with a fragment comprising the entire glucoamylase gene and short regions of the *cbh1* promoter and terminator. There was no evidence of any rearrangement of the expression cassettes or any insertion of the expression cassettes at alternative sites in the genome of the production strain during commercial fermentation runs. It is concluded that the introduced DNA has been stably integrated into the host's genome.

## 3.3 Safety of glucoamylase

### 3.3.1 History of safe use

Glucoamylase produced by GM *T. reesei* TrGA-LOVMC2#3 (from host strain M1-1.1), containing the glucoamylase gene from *T. reesei* has been approved in France since 2018 and Denmark since 2016 for use in brewing, baking, alcohol production, starch industry and glucose syrup production.

In 2019 FSANZ assessed and recommended approval of four other enzymes from *T. reesei* host strain M1-1.1 Several glucoamylase enzymes from other GM and non-GM microbial sources (*A. niger*; *A. oryzae*, *R. delemar*; *R. oryzae* and *R. niveus*) are also currently permitted as processing aids in Schedule 18 of the Code.

### 3.3.2 Bioinformatic assessment of enzyme toxicity

A BLAST search for homology of the glucoamylase amino acid sequence against the [UniProt Protein Knowledge database](#) was performed with a threshold E-value of 0.1. The majority of matches were glucoamylase, with none of the top 1000 database matches being annotated as a toxin or venom. Therefore, the glucoamylase sequence does not share homology with a known toxin or venom sequence.

### 3.3.3 Toxicology studies in animals

The applicant provided OECD guideline and GLP (Good Laboratory Practice) compliant 13-week oral toxicity and mutagenicity studies for two closely-related enzymes from host strain M1-1.1, (a glucoamylase and a trehalase).

#### 3.3.3.1 Glucoamylase

The glucoamylase enzyme that is the subject of this application has a high degree of amino acid homology to that assessed previously by JECFA (WHO, 2013). JECFA identified a NOAEL (No Observed Adverse Effect Level) of 166.4 mg/kg bw/day TOS (total organic solids), the highest dose tested, for the glucoamylase preparation. Glucoamylase was not genotoxic in an OECD 471 (1997) bacterial reverse mutation assay or an OECD 473 (1997) chromosomal aberration test.

#### 3.3.3.2 Trehalase

*90-day repeated dose oral toxicity study in rats (E.I. du Pont de Nemours and Company, 2018). Regulatory status: GLP; conducted in accordance with OECD Guideline 408 (1998)*

The test item in this study was trehalase from *T. reesei*. Sprague Dawley rats of the Crl:CD®(SD) strain (10/sex/group, approximately 6 weeks old) were administered trehalase at doses of 0, 250, 500 or 1000 mg/kg bw/day TOS. The vehicle and negative control article was deionized water. Clinical signs were monitored daily with more detailed observations performed weekly. A functional observational battery including an open field test, grip strength and stimuli-induced tests was performed during the last two weeks of treatment. Bodyweight and food consumption were recorded weekly. Ophthalmoscopy was performed on all rats prior to the start of treatment and on all control and high dose group animals prior to termination of treatment. Blood and urine were collected from all animals during Week 13, the blood samples were evaluated for haematology (including assessment of coagulation factors) and clinical chemistry. At necropsy, bone marrow smears, from the femur and sternum, were collected from each animal, followed by a macroscopic examination, selected organ weights were recorded and a comprehensive range of organs and tissues was

preserved for histopathological examination. Histopathological examination was confined to all control and high-dose animals, plus any macroscopically abnormal tissues from animals in other treated groups.

Samples of dose formulations were taken in Weeks 1, 6 and 11 and analysed for total protein content and found to be suitable for study.

There were no deaths during the study and no toxicologically important effects on clinical observations, behaviour in an arena, sensory reactivity tests, grip strength, motor activity measured, bodyweight gains, food consumption, food efficiency, ophthalmic findings, haematology, blood chemistry, organ weights or gross pathology findings.

The NOAEL in this study was 1000 mg/kg bw/day TOS, the highest dose tested.

### **3.3.4 Genotoxicity assays**

The test item for these studies was trehalase from *T. reesei*,

*Bacterial reverse mutation assay (E.I. du Pont de Nemours and Company, 2017) Regulatory status: GLP; in accordance with OECD Guideline 471 (1997) and Method B13/14 of Commission Directive 2000/32/EC or Commission Regulation (EC) No. 440/2008 B13/14 (2008) and EPA Health Effects Test Guidelines OPPTS 870.5100 (1998).*

Test concentrations were reported in terms of the total protein content of the test item trehalase (270.74 mg/mL). The vehicle and negative control was sterile water. Test systems for the assay were *Salmonella typhimurium* strains TA100, TA98, TA1537, TA1535 and *Escherichia coli* WP2uvrA. Following a preliminary toxicity assay with all strains, concentrations of 333, 667, 1000, 3333 and 5000 µg/plate trehalase were used in two independent main tests, both conducted in the presence and absence of metabolic activation (S9 mix). All assays were conducted in triplicate.

There was no evidence of mutagenicity, cytotoxicity or precipitation at any of the dose levels investigated. Results obtained with the negative and positive controls were compatible with historical control values for the laboratory, confirming the validity of the assays. It was concluded that under the conditions of this study, the trehalase did not cause a positive mutagenic response with any of the tester strains in either the presence or absence of metabolic activation.

*Chromosomal aberration assay in cultured human lymphocytes (E.I. du Pont de Nemours and Company, 2017) Regulatory status: GLP; conducted in accordance with OECD TG 473 (2014/2016), Commission Regulation (EC) No 440/2008 B.10 and US EPA Health Effects Test Guidelines OPPTS 870.5375 (1998)*

Two experiments were conducted. In experiment I cells were exposed to the test substance in the presence or absence of metabolic activation (S9 mix) for 4 hours. In experiment II cells were exposed to the test substance for 22 hours without S9 and 4 hours in the presence of S9. Concentrations of up to 5000 µg/mL were tested in all experiments. Concentrations were expressed in terms of the protein content of the test item (270.74 mg/mL). All cultures were harvested 22 hours after the start of treatment. Cells were cultured in duplicate.

No precipitation or cytotoxicity was observed at any test concentration. No statistical or biological significant increases in the percentage of cells with chromosomal aberrations were observed compared with controls. The percentage of cells with chromosomal aberrations was significantly increased in all the positive control cultures, confirming the validity of the assay.

It was concluded that trehalase from *T. reesei*, did not cause chromosomal aberrations in cultured human lymphocytes under the conditions of this study.

### 3.3.5 Potential for allergenicity

The applicant performed several searches for homology of the amino acid sequence of the glucoamylase enzyme that is the subject of this application to known allergens using the [Allergen Online database](#) of the University of Nebraska's Food Allergy Research and Resource Program (FARRP). The following searches were conducted:

- A search for full-length sequence alignment for matches of > 35% identity
- A search using a sliding window of 80 amino acid stretches for identities > 35%
- A search for exact matches of 8 contiguous amino acids

The search for full-length sequence alignment found one match with more than 35% identity to the glucoamylase sequence: a glycoside hydrolase from *Schizophyllum commune*, also known as Sch. c. 1.010. Matches to this allergen were also identified in the searches for 80 amino acid stretches with > 35% identity to known allergens and for exact matches of 8 contiguous amino acids. *S. commune* (splitgill mushroom) is eaten in Africa, Asia, the Indian subcontinent, and Central America but is not associated with reports of food allergy.

The JECFA review (WHO, 2013) of the closely-related glucoamylase from strain *T. reesei* 70H2-TrGA#32-9 also reported >35% homology over a 80 amino acid window, to proteins from *Schizophyllum commune* and *P. chrysogenum*. JECFA acknowledged neither protein were identified as food allergens in the WHO-IUIS list and after further analysis, including using a search for six contiguous amino acid sequences, concluded oral intake of the glucoamylase is not anticipated to pose a risk of allergenicity.

In summary the available evidence, taken together with the very low levels of glucoamylase expected to be present in the final foods following use, indicates that the risk of food allergy from use of glucoamylase from *T. reesei* is likely to be negligible.

The applicant has indicated that glucose, from wheat, may be included in the fermentation medium and the final formulation.

## 4 Discussion

*T. reesei* has a long history of safe use as a source of enzyme processing aids, including several that are already permitted in the Code. This fungus is neither toxigenic nor pathogenic. Characterisation of the GM production strain (TrGA-LOVMC2#3) confirmed both the presence and stable inheritance of the inserted glucoamylase gene.

Strain#4, which is the production strain for the application glucoamylase (*T. reesei* TrGA-LOVMC2#3), was also shown to be neither toxigenic nor pathogenic.

The glucoamylase enzyme (that is the subject of this application) has a high degree of amino acid homology to that assessed previously by JECFA (WHO, 2013). JECFA identified a NOAEL of 166.4 mg/kg bw/day TOS, the highest dose tested, for the glucoamylase preparation. Glucoamylase was not genotoxic in a bacterial reverse mutation assay or a chromosomal aberration test.

A closely related trehalase enzyme from host strain M1-1.1 was not genotoxic *in vitro* and caused no adverse effects in a 13-week repeat dose toxicity study in rats. The NOAEL was

1000 mg/kg bw/day TOS, the highest dose tested. The applicant's estimated theoretical maximal daily intake (TMDI) based on the proposed use pattern is 3.18 mg/kg bw/day TOS. A comparison of this value with the NOAEL of the closely-related trehalase (1000 mg/kg/day TOS) enzyme indicates that the Margin of Exposure between the NOAEL and TMDI is more than 300.

Bioinformatic analysis indicated that the enzyme has no significant homology with any known toxins or food allergens, and is unlikely to pose an allergenicity or toxicity concern. The applicant has indicated that glucose, from wheat, may be included in the fermentation medium and the final formulation.

## **5 Conclusion**

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.

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