An Alpha-glucosidase Enzyme
from A Recombinant Strain of *Trichoderma reesei*

PROCESSING AID APPLICATION

Food Standards Australia
New Zealand

Applicant: DUPONT AUSTRALIA PTY LTD
Submitted by: AXIOME PTY LTD

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EXECUTIVE SUMMARY:

DuPont Industrial Biosciences (IB) is seeking approval for an alpha-glucosidase (EC 3.2.1.20) enzyme for manufacturing of potable alcohol, lysine, lactic acid, monosodium glutamate (MSG) and other biochemicals, production of isomalto-oligosaccharides (IMO) syrup and other sweeteners. The enzyme is designated as "Alpha-glucosidase" throughout the dossier.

The enzyme Alpha-glucosidase is derived from a selected non-pathogenic, non-toxigenic strain of *Trichoderma reesei* which is genetically modified to overexpress the alpha-glucosidase gene from *Aspergillus niger*.

Alpha-glucosidase catalyses decomposition or hydrolysis reaction, which catalyses the hydrolysis of terminal, non-reducing (1→4)-linked α-D-glucose residues with release of α-D-glucose. This reaction catalyses the conversion of non-fermentable sugars in molasses such as raffinose and stachyose to sucrose, galactose, glucose and fructose, which can then be fermented into alcohol. The same reaction is also used in yeast fermentation to manufacture potable alcohol, organic acids (e.g. lactic acid, citric acid), MSG and other biochemicals.

Alpha-glucosidase also catalyzes a synthetic or transfer reaction, which transfers a glucosyl residue from the substrate (maltose) and form oligosaccharides having alpha-D 1,6 linkages like isomaltose, panose, isomaltotriose and higher branched oligosaccarides together with varying amounts of glucose. The main intention of the synthetic or transfer reaction of Alpha-glucosidase is to facilitate the production of IMO syrups from starch. This activity of Alpha-glucosdiase was also referred to as transglucosidase (EC number 2.4.1.24, CAS number 9030-12-0) in some submissions and approvals.

In all of these applications, Alpha-glucosidase will be used as a processing aid where the enzyme is either not present in the final food or present in insignificant quantities having no function or technical effect in the final food.

To assess the safety of the Alpha-glucosidase for use in these applications, DuPont IB vigorously applied the criteria identified in the guidelines as laid down by Food Standards Australia New Zealand (FSANZ) and U.S. Food and Drug Administration (FDA) utilizing enzyme toxicology/safety data, the safe history of use of enzyme preparations from *T. reesei* and of other alpha-glucosidase enzymes in food, the history of safe use of the *T. reesei* production organism for the production of enzymes used in food, an allergenicity evaluation, and a comprehensive survey of the scientific literature.

In addition, different endpoints of toxicity were investigated, and the results are evaluated and assessed in this document. Alpha-glucosidase is non-hazardous based on acute oral studies. In genotoxicity studies, Alpha-glucosidase is not mutagenic, clastogenic or aneugenic. Daily oral administration of Alpha-glucosidase up to and including a dose level of 63.64 mg total protein/kg bw/day or 77.2 mg total organic solid (TOS)/kg bw/day does not result in any manifestation of systemic, hematologic, or histopathologic adverse effects.

Based on a worst-case scenario that a person is consuming Alpha-glucosidase from the biochemical and sweetener production process, the calculated Theoretical Maximum Daily Intake (TMDI) will be 0.443 mg TOS/kg body weight/day. This still offers a 174× fold margin of safety.

Based on the results of safety studies and other evidence, Alpha-glucosidase has been demonstrated as safe for its intended applications and at the proposed usage levels.

Approval of this application would provide manufacturers and/or consumers with benefits of facilitating the production of sweeteners, potable alcohols and biochemicals, lowering the manufacturing cost, and improving quality of final foods.

General information

1.1 Applicant details

(a) Applicant:

This application is made by Axiome Pty Ltd on behalf of DuPont Australia Pty Ltd

(b) Company:

DuPont Australia Pty Ltd

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(Danisco Singapore Pte Ltd is a subsidiary of E. I. du Pont de Nemours and Company)

(e) Email Address:

See above

(f) Nature of Applicants Business:

DuPont Australia Pty Ltd – A subsidiary of E. I. du Pont de Nemours and Company, manufacturer/marketer of specialty food ingredients, food additives and food processing aids.

Axiome Pty Ltd – regulatory & scientific affairs consultants

(g) Details of Other Individuals etc.:

No other individuals, companies or organizations are associated with this application.

1.2 Purpose of the application

This application seeks to modify Schedule 18 to Standard 1.3.3 Processing Aids to permit the use of a new *Processing Aid*, subject of this application. The intended use of the processing aid includes manufacturing of biochemicals such as monosodium glutamate (MSG), organic acids, potable alcohol, and sweeteners (e.g. IMO).

This application is made solely on behalf of DuPont Industrial Biosciences (IB), the manufacturer/marketer of the *Processing Aid*. When approved, the *Processing Aid* would be available for use by any food manufacturer in Australia and New Zealand.

Currently no alpha-glucosidase from *T. reesei* is permitted as a Processing Aid, however other enzymes including Cellulase, Endo-1,4-beta-xylanase, β-Glucanase, Hemicellulase multicomponent enzyme, Polygalacturonase or Pectinase multicomponent enzyme, from *T. reesei* are listed in Schedule 18 section S18-4(5) as permitted enzymes. Approval of this application would provide food processors with a new enzyme preparation offering the benefits and advantages as discussed in Section 2.3 and Appendix A.

1.3 Justification for the application

1.3.1. Regulatory Impact Information

A. Costs and Benefits of the application

Alpha-glucosidase is an enzyme produced by submerged fermentation of *T. reesei* carrying the gene encoding the alpha-glucosidase from *Aspergillus niger.* The enzyme is characterized as alpha-D-glucoside glucohydrolase (EC 3.2.1.20, CAS 9001-42-7). A collection of information detailed in Section 3 supports the safety of the production organism and the enzyme for use in the applications outlined in Section 4.

Alpha-glucosidase catalyzes a decomposition or hydrolysis reaction, which can be used to facilitate manufacture of potable alcohol, organic acids, MSG and other biochemicals. Alpha-glucosidase also catalyzes synthetic or transfer reaction to facilitate the production of IMO syrups and other sweeteners from starch.

More information on the benefit of this enzyme can be found in Section 2.2 and Appendix A.

Enzyme preparations are widely used as processing aids in the manufacture of food products. Currently no alpha-glucosidase from *T. reesei* is permitted as a Processing Aid. Approval of this application would provide food processors with a new enzyme preparation offering the benefits and advantages as discussed previously.

B. Impact on international trade

The inclusion of alpha-D-glucoside glucohydrolase from *A. niger* expressed in *T. reesei* in the Australia New Zealand Food Standards Code as a processing aid may promote international trade on products produced with this enzyme product and reduce technical barriers to trade.

1.4. Support for the application

No marketing or promotional activities have been undertaken for Alpha-glucosidase derived from *T. reesei* containing the gene for alpha-glucosidase from *A. niger* in the Australia/New Zealand market. Hence at this stage, no requests from food manufacturers are provided in support of this application. However, the need and justification for use of the processing aid are discussed in Section 1.3, and it is anticipated that support from the food processing industry will be submitted during the period for public comment on the application Draft Regulatory Measure/Assessment Report.

1.5. Assessment Procedure

This application seeks to modify Schedule 18 to Standard 1.3.3 Processing Aids to permit the use of a Processing aid that is currently not permitted. Based on guidance in the Application Handbook, DuPont IB considers General Procedure Level 1 (up to 350 hours) to be the appropriate procedure for assessment of the application.

1.6. Confidential Commercial Information (CCI)

Certain (identified) technical and manufacturing information included in Appendix B1, B2, B3, B4, B5, Appendix E and other information including amino acid sequences labelled with Confidential Commercial information is regarded by the applicant as **Confidential Commercial Information** and is provided in the application strictly on this basis. This information is the result of a significant research and development effort and investment by the applicant; it is not in the public domain and is considered as either proprietary or commercially sensitive. It would be disadvantageous to the applicant if this information were released into the public domain.

1.7. Exclusive Commercial Capturable Benefit (ECCB)

According to Section 8 of the FSANZ Act, this application is not expected to confer Exclusive Capturable Commercial Benefit (ECCB).

1.8. International and other National Standards

Refer to Appendix D for further details

1.8.1 Codex Standards

Alpha-glucosidase produced by *T. reesei* has not been reviewed by JECFA; there is no specific Codex Standard relevant to this application.

1.8.2 International Legislation

Alpha-glucosidase derived from *T. reesei* carrying the gene encoding the alpha-glucosidase gene from *A. niger* has been determined to be Generally Recognized as Safe (GRAS) in the United States as a food processing aid to manufacture potable alcohol, organic acids and MSG, and as transglucosidase for production of IMO and potable alcohol from molasses.

1.9. Statutory declaration

I,

of Australia, regulatory affairs consultant:

make the following declaration under the *Statutory Declarations Act 1959:*

1) The information provided in this application fully sets out the matters required

2) The information provided in this application is true to the best of my knowledge and belief

3) No information has been withheld which might prejudice this application, to the best of my knowledge and belief

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

Signature: _____

Declared at _____ on _____ of _____

Before me,

Signature: _____

1.10. Checklist

CHECKLIST FOR STANDARDS RELATED TO SUBSTANCES ADDED TO FOOD

This checklist will assist you in determining if you have met the information requirements as detailed in the Application Handbook. Section 3.1 – General Requirements is mandatory for all applications. Sections 3.3.1-3.3.3 are related to the specifics of your application and the information required is in addition to section 3.1.

General Requirements (3.1)

☒ Form of application	☒ Assessment procedure
☒ Applicant details	☒ Confidential Commercial Information
☒ Purpose of the application	☒ Exclusive Capturable Commercial Benefit
☒ Justification for the application	☒ International standards
☒ Information to support the application	☒ Statutory Declaration

Food Additives (3.3.1)

☐ Support for the application	☐ Analytical detection method
☐ Nature and technological function information	☐ Toxicokinetics and metabolism information
☐ Identification information	☐ Toxicity information
☐ Chemical and physical properties	☐ Safety assessments from international agencies
☐ Impurity profile	☐ List of foods likely to contain the food additive
☐ Manufacturing process	☐ Proposed levels in foods
☐ Specifications	☐ Percentage of food group to contain the food additive
☐ Food labelling	☐ Use in other countries (if applicable)

Processing Aids (3.3.2)

☒ Support for the application	☒ Information on enzyme use on other countries (enzyme only)
☒ Type of processing aid	☒ Toxicity information of enzyme (enzyme only)
☒ Identification information	☒ Information on source organism (enzyme from micro-organism only)
☒ Chemical and physical properties	☒ Pathogenicity and toxicity of source micro-organism (enzyme from micro-organism only)
☒ Manufacturing process	☒ Genetic stability of source organism (enzyme from micro-organism only)
☒ Specification information	☒ Nature of genetic modification (PA from GM micro-organism only)
☐ ~~Industrial use information (chemical only)~~	☒ List of foods likely to contain the processing aid

- ☐ Information on use in other countries (chemical only)
- ☐ Toxicokinetics and metabolism information (chemical only)
- ☐ Toxicity information (chemical only)
- ☐ Safety assessments from international agencies (chemical only)
- ☐ Anticipated residue levels in foods
- ☐ Percentage of food group to use processing aid
- ☐ Information on residues in foods in other countries (if available)

Nutritive Substances (3.3.3)

- ☐ Support for the application
- ☐ Identification information
- ☐ Information on chemical and physical properties
- ☐ Impurity profile information
- ☐ Manufacturing process information
- ☐ Specification information
- ☐ Analytical detection method
- ☐ Proposed food label
- ☐ Toxicokinetics and metabolism information
- ☐ Animal or human toxicity studies
- ☐ Safety assessments from international agencies
- ☐ List of food groups or foods likely to contain the nutritive substance
- ☐ Proposed maximum levels in food groups or foods
- ☐ Percentage of food group anticipated to contain nutritive substance
- ☐ Food consumption data for new foods
- ☐ Information on use in other countries
- ☐ Food consumption data for foods with changed consumption patterns
- ☐ Nutritional purpose
- ☐ Need for nutritive substance in food
- ☐ Demonstrated potential deficit or health benefit
- ☐ Consumer awareness and understanding
- ☐ Actual or potential behaviour of consumers
- ☐ Demonstration of no adverse affects to any population groups
- ☐ Impact on food industry
- ☐ Impact on trade

2. Technical information

Please refer to Appendix A for further details

2.1. Type of processing aid

The Alpha-glucosidase enzyme is an enzyme produced by submerged fermentation of *T. reesei*, carrying the alpha-glucosidase gene from *A. niger*.

This Processing Aid falls into the category "Enzymes of microbial origin" from the Food Standard Code section 1.3.3-6 Enzymes.

2.2. Identity

2.2.1 Chemical/Common Name:

The systematic name of the principle enzyme activity is alpha-D-glucoside glucohydrolase. Other names used are acid maltase, glucoinvertase, glucosidosucrase, lysosomal alpha-glucosidase, maltease, myltase-glucoamylase.

- EC number: 3.2.1.20
- CAS number: 9001-42-7

The enzyme described in this dossier was also identified historically as Transglucosidase (EC 2.4.1.24), with synonyms oligoglucan-branching glycosyltransferase; 1,4-alpha-D-glucan 6-alpha-D-glucosyltransferase; T-enzyme; D-glucosyltransferase; and 1,4-alpha-D-glucan:1,4-alpha-D-glucan(D-glucose) 6-alpha-D-glucosyltransferase.

Biological source: The Alpha-glucosidase enzyme is an enzyme produced by submerged fermentation of *Trichoderma reesei*, carrying the alpha-glucosidase gene from *Aspergillus niger*.

2.2.2 Marketing Name of the Processing Aid:

Some example marketing name of Alpha-glucosidase include FERMENZYME® TL, and FERMENZYME® TL FG.

2.2.3 Molecular and Structural Formula:

Alpha-glucosidase is a protein. The amino acid sequence is known. Please refer to Appendix E.

2.3. Chemical and physical properties

The decomposition or hydrolysis reaction of Alpha-glucosidase catalyses the hydrolysis of terminal, non-reducing (1→4)-linked α-D-glucose residues with release of α-D-glucose. This reaction catalyzes the conversion of non-fermentable sugars in molasses such as raffinose and stachyose to sucrose, galactose, glucose and fructose, which can then be fermented into alcohol. The same reaction is also used in yeast fermentation to manufacture potable alcohol and organic acids (e.g. lactic acid, citric acid) and MSG.

The synthetic or transfer reaction transfers a glucosyl residue from the substrate (maltose) and form oligosaccharides having alpha-D 1,6 linkages like isomaltose, panose, isomaltotriose and higher branched oligosaccarides together with varying amounts of glucose. These oligosaccharides are having an altered digestibility which have health benefits. The main intention of the synthetic or transfer reaction of Alpha-glucosidase is to facilitate the production

of IMO syrups from starch. However, its use has also some beneficial effects on the final syrup, such as: mildly sweet (about half as sweet as sucrose), lower viscosity than maltose syrups, preventing staling (retrogradation) of starchy foods and retaining of a suitable moisture level in foods which helps to control microbial growth. Historically, the enzyme is also described as transglucosidase in the synthetic or transfer reaction. Transglcuosidase is still used in some of the appendices and study reports attached in this dossier to describe the same enzyme.

In all of these applications, the enzyme preparation will be used as a processing aid where the enzyme is not present or active in the final food or present in negligible amounts with no technical function in the final food.

Appearance:

Brown liquid.

Substrate specificity:

In the decomposition or hydrolysis reaction, alpha-glucosidase hydrolysis of terminal, non-reducing (1->4)-linked alpha-D-glucose residues with release of alpha-D-glucose.

In the synthetic or transfer reaction, Alpha-glucosidase hydrolyses and transfers an alpha-D-glucosyl units of oligosaccharides and convert 1,4 glucosidic linkage to 1,6 glucosidic linkages. Transfer occurs most frequently to HO-6 (the hydroxy group at the 6-position), producing isomaltose from D-glucose, and panose from maltose.

Activity:

In the decomposition or hydrolysis reaction, the activity of the Alpha-glucosidase is defined in U (units) /g. This activity is measured based on the ability of alpha-glucosidase enzyme to catalyze the hydrolysis of *p*-nitrophenyl-alpha-D-glucopyranoside (PNPG) to glucose and *p*-nitrophenol. At an alkaline pH, the nitrophenol forms a yellow color that is proportional to alpha-glucosidase activity and is monitored at 420nm via the use of an enzyme standard.

In the synthetic or transfer reaction, the activity of the Alpha-glucosidase is defined in TGU (Transglucosidase Unit). TGU is defined as the amount of enzyme which will produce one micromole of trisaccharide per minute under assay conditions.

Temperature optimum:

Approximately 58-70°C, with activity observed from 30°C until 90°C.

Thermal stability:

The enzyme is relatively stable for 30 minutes at 60°C, while it is inactivated after 30 minutes of incubation at 71°C.

pH optimum:

Approximately below pH 5.6.

pH stability:

The enzyme exhibits activity at pH <8.3.

Interaction of the enzyme with different foods:

The Alpha-glucosidase enzyme preparation will be used as a processing aid where the enzyme is not present or active in the final food or present in negligible amounts with no technical function in the final food.

Nutritional implication:

Alpha-glucosidase is a protein and any residual amounts remaining in food consumed would accordingly have the same nutritional value. However, the use levels of Alpha-glucosidase are very low, and as with other enzymes that are currently approved and used as Processing Aids use of this preparation would not have any nutritional significance.

2.4. Manufacturing process

The enzyme is produced by a submerged fermentation process using appropriate substrate and nutrients. When fermentation is complete, the biomass is removed by centrifugation/filtration. The remaining fermentation broth containing the enzyme is filtered and concentrated. The concentrated enzyme solution is then standardised and stabilised with diluents. Finally, a polish filtration is applied.

Full details on the raw materials used for the production are provided in Appendix E. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

The production of Alpha-glucosidase is monitored and controlled by analytical and quality assurance procedures that ensure that the finished preparation complies with the specifications and is of the appropriate quality for use as a processing aid in food processing applications.

2.5. Specification for identity and purity

Impurity profile:

Appropriate GMP controls and processes are used in the manufacture of Alpha-glucosidase to ensure that the finished preparation does not contain any impurities of a hazardous or toxic nature. The specification for impurities and microbial limits are as follows:

Metals:	
Lead	less than 5 mg/kg
Microbiological:	
Total viable count	less than 50,000 CFU/ml
Total coliforms	less than 30 CFU/ml
E. coli	absent in 25ml
Salmonella	absent in 25ml
Antibiotic activity	Absent in 1ml of sample
Production strain	Negative in 1ml
Physical properties:	
Appearance	brown liquid

Standard for identity:

Alpha-glucosidase meets the specifications laid down by the Joint FAO/WHO Expert Committee on Food Additives and the Food Chemicals Codex.

2.6. Allergenicity of the enzyme:

An allergen statement is given in Appendix A. Refer to Appendix B for additional information on the safety of the enzyme as to its allergenicity potential.

3. Safety

Refer to Appendix B for further details

3.1. Use of the enzyme as a food processing aid in other countries

Enzyme products are developed for a specific function, i.e. to catalyze a specific chemical reaction. That reaction determines the IUBMB classification. Enzyme variants may be selected to have a better performance of that function under the specific conditions of the application (e.g. temperature or pH). Enzymes of a certain IUBMB classification share conserved structural elements, called domains, which are needed for their specific function. As such the enzymes of our approval procedures do resemble those already permitted by FSANZ both in function and in structure.

Figure 1 below shows an example of natural variation of alpha-amylases. The same holds for any other enzyme type. While significant differences in sequence amongst the various species exist, they all catalyze the same reaction and therefore fit under the same IUBMB entry. There will also be natural variation within one species. All this also applies to the enzymes under the current approval procedures by FSANZ:

% amino acid sequence identity	*B. amyloliquefaciens*	*B. licheniformis*	*G. stearothermophilus*	*A. niger*	*A. oryzae*	*Z. mays*	*O. sativa*	*H. vulgare*	*P. vulgaris*	*H. sapiens*
Bacillus amyloliquefaciens	100									
Bacillus licheniformis	80	100								
Geobacillus stearothermophilus	65	65	100							
Aspergillus niger	21	21	22	100						
Aspergillus oryzae	23	24	24	66	100					
Zea mays (corn)	24	26	25	28	27	100				
Oryza sativa (rice)	25	27	25	27	26	89	100			
Hordeum vulgare (barley)	25	23	24	25	28	70	69	100		
Phaseolus vulgaris (bean)	26	27	25	24	27	67	65	64	100	
Homo sapiens (human)	25	33	29	22	28	23	22	23	24	100

α-amylases in nature have divergent

amino acid sequences but have the same catalytic activity and IUBMB number

Figure 1. Variation of enzymes in nature.

The expressed mature enzyme amino acid sequence of the Alpha-glucosidase shows a clear conserved Glyco_hydro_31 superfamily sequence domain, and a clear conserved YicI protein domain, characteristic for alpha-glucosidase family GH31 activities.

The Alpha-glucosidase enzyme is one of the approved alpha-glucosidase enzymes on Schedule 18 of the ANZ Food Standards Code, i.e. the *Aspergillus niger* one. The subject of this application, the enzyme protein is expressed from *Trichoderma reesei*. The identity between the FSANZ approved alpha-glucosidases (*A. niger* and *A. oryzae*) is 78.3%. It is good to realize that the alpha-glucosidase sequences within one species can show strain dependent amino acid sequence variability, i.e. an alignment of two *A. niger* alpha-glucosidase amino acid sequences showed that these were 99.5 - 100% identical.

Alpha-glucosidase enzyme derived from *T. reesei*, carrying the alpha-glucosidase gene from *A. niger* has been determined to be GRAS in the United States, and been used for IMO production since 2009 in the U.S and for production of organic acids and potable alcohol since 2014. There have not been any adverse events reported since Alpha-glucosidase has been in commercial use in these countries.

Please refer to section 1.8 and Appendix D for details on the different approval procedures in the countries listed above.

3.2. Toxicity of the enzyme

Toxin homology study

A BLAST search for homology of the Alpha-glucosidase sequence against the UniProt database was performed. The majority of matches were alpha-glucosidases, with none of the top 1000 database matches being annotated as either toxin or venom. In addition, a specific BLAST search for homology of the mature Alpha-glucosidase sequence was performed against the Uniprot animal toxin database. This yielded no matches. Therefore, the Alpha-glucosidase sequence does not share homology with a known toxin or venom sequence.

Please refer to Appendix B for details.

Safe Strain Lineage concept

The Safe Strain Lineage concept has been discussed by Pariza and Johnson (2001) in their publication on the safety of food enzymes and is commonly utilized by enzyme companies in the determination of the safety of their products for specific uses, as appropriate.

The primary issue in evaluating the safety of a production strain is its toxigenic potential, specifically the possible synthesis by the production strain of toxins that are active via the oral route. The toxigenic potential of the production organism is confined to the Total Organic Solid (TOS) originating from the fermentation.

As the toxicological evaluation is based on the TOS originating from fermentation of the production organism, studies conducted on strains from the Safe Strain Lineage can support other production strains pertaining to this same Safe Strain Lineage.

Although *T. reesei* is scientifically determined by DuPont IB as a Safe Strain Lineage, the food enzyme object of the current dossier is supported by toxicological studies on the specific food enzyme object of this dossier. The toxicological studies on production organism of Alpha-glucosidase (*T. reesei* Morph TG #626) are thus one of the pillars supporting the DuPont IB *T. reesei* Safe Strain Lineage. The position of the food enzyme in the DuPont IB *Trichoderma reesei* Safe Strain Lineage is presented in Appendix B2.

Toxicological testing

To assess the safety of Alpha-glucosidase, different endpoints of toxicity were investigated at Harlan Laboratories (Switzerland) and are evaluated and assessed in this document:

Studies:

Acute Oral Toxicity Study in Rats
Bacterial Reverse Mutation Assay – Ames assay

In vitro Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes
A 90-days Oral Toxicity (Gavage) Study in Wistar Rats

The safety of Alpha-glucosidase from *T. reesei* strain Morph TG #626 as a food processing aid is assessed in a battery of toxicology studies investigating its acute oral, mutagenic and systemic toxicity potential. Alpha-glucosidase is not acutely toxic. A battery of genotoxicity assays was conducted and under the conditions of these assays. Alpha-glucosidase is not a mutagen, a clastogen, or an aneugen. Daily administration of Alpha-glucosidase by gavage for 18 consecutive weeks did not result in overt signs of systemic toxicity. A NOAEL is established at 63.64 mg total protein/kg bw/day corresponding to 77.2 mg TOS/kg bw/day.

A summary of the results of the studies can be found in Appendix B.

In addition, safety was further assessed according to the decision tree in the Pariza-Johnson guidelines (2001) for assuring the safety of a new enzyme preparation.

3.3 Information on the source micro-organism

The production organism of Alpha-glucosidase, strain Morph TG #626, is a strain of *T. reesei* which has been genetically modified by DuPont IB to overexpress an alpha-glucosidase gene from *A. niger*.

T. reesei has a long history of safe use in industrial scale enzyme production. The safety of this species as an industrial enzyme producer has been reviewed by Nevalainen *et al*. (1994), Blumenthal (2004) and Olempska-Beer et al. (2006). The organism is considered non-pathogenic for humans and does not produce fungal toxins or antibiotics under conditions used for enzyme production. It is generally recognized as a safe production organism and is the source organism of a range of enzyme preparations that are used as processing aids in the international food and feed industries. It is also considered as suitable for Good Industrial Large Scale Practice (GILSP) worldwide and meets the criteria for a safe production microorganism as described by Pariza and Johnson (2001).

Full details of the gene and recombinant microorganism are provided in Appendix E. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

3.4. Pathogenicity and toxicity of the source micro-organism

Trichoderma reesei was first isolated from nature in 1944. The original isolate, QM6a (Mandels and Reese, 1957), and its subsequent derivatives have been the subject of intense research due to their usefulness in the production of cellulases. In the 1980s, it was suggested that *Trichoderma reesei* be placed into synonymy with *Trichoderma longibrachiatum* (Bissett 1984). Subsequent evidence pointed out that the two species are not identical (Meyer *et al*. 1992) even though several regulatory jurisdictions still use both names interchangeably. The proposal by Khuls *et al*. (1996) that *Trichoderma reesei* was a clonal derivative of *Hypocrea jecorina* is being accepted by more and more people in the science community, and the US National Center for Biotechnology Information (NCBI) refers to *Trichoderma reesei* as the anamorph of *Hypocrea jecorina* and no longer includes it in the genus *Trichoderma*. Therefore, *Trichoderma reesei*, *Trichoderma longibrachiatum,* and *Hypocrea jecorina* may appear in different documents and national positive lists, but for historical reasons they refer to essentially the same microorganism species.

A literature search was conducted on August 28, 2017 using the searching term "*Trichoderma reesei*" and "food safety OR toxin OR toxicology OR pathogen" on PubMED resulting in 43 records. A review of the literature search uncovered no reports that implicate *Trichoderma reesei* in any way with a disease situation, intoxication, or allergenicity among healthy adult human and animals. The species is not present on the list of pathogens used by the EU (Council Directive 90/679/EEC, as amended) and major culture collections worldwide. It is classified as Biosafety Level 1 (BSL1) microorganism by the American Type Culture Collection (ATCC) based on assessment of the potential risk using U.S. Department of Public Health guidelines with assistance provided by ATCC scientific advisory committees. BSL1 microorganisms are not known to cause diseases in healthy adult humans.

Two authors reported the isolation from *T. reesei* strain QM 9414 a peptaibol compound that exhibited antibiotic activity (Brukner and Graf 1983). Their work was confirmed by another group that found evidence of peptaibol production in two other *T. reesei* strains (Solfrizzo *et al.* 1994). However, peptaibols' antibiotic activity is clinically useless and commercially irrelevant, and the growth conditions under which the compounds were produced are very different from those in enzyme manufacturing.

Strain QM 9414 and its derivatives have been safe producers of commercial cellulase enzyme preparations for food applications. The industrial enzyme preparations are still confirmed by the enzyme manufacturers not to have antibiotic activity according to the specifications recommended by JECFA (2006).

T. reesei has a long history of safe use in industrial scale enzyme production. The safety of this species as an industrial enzyme producer has been reviewed by Nevalainen *et al*. (1994) and Blumenthal (2004). The organism is considered non-pathogenic for humans and does not produce fungal toxins or antibiotics under conditions used for enzyme production. It is generally considered a safe production organism and is the source organism of a range of enzyme preparations that are used as processing aids in the international food and feed industries. It is listed as a safe production organism for cellulases by Pariza and Johnson (2001) and Olempska-Beer *et al.* (2006), and various strains have been approved for the manufacture of commercial enzyme preparations by Food Standards Australia New Zealand (FSANZ), and internationally, for example, in Canada (Food and Drugs Act Division 16, Table V), the United States (21CFR § 184.1250), Mexico, Brazil, France, Denmark, China, and Japan.

3.5. Genetic stability of the source organism

The parental strain of the production strain *Trichoderma reesei* QM6a and its derivatives have been used for industry scale enzyme manufacturing for decades by DuPont IB and its parental companies, and have demonstrated stable enzyme expression even at large scale fermentation. Please also refer to Appendix B2 for list of example enzyme preparations produced using QM6a and its derivatives. Furthermore, the production strain has demonstrated to be completely stable after industrial scale fermentation, judged by alpha-glucosidase production derived from the integrated expression cassettes. Refer also section 3.6.

3.6. Method used in the genetic modification of the source organism

The production organism of the alpha-glucosidase preparation, the subject of this submission, is *T. reesei* strain Morph TG #626. It is derived by recombinant DNA methods from strain RL-P37. The purpose of this genetic modification is to express alpha-glucosidase from *A.niger*. RL-P37, a commercial production strain, is derived, as a result of several classical mutagenesis steps, from

the well-known wild-type strain QM6a. Virtually all strains used all over the world for industrial cellulase production today are derived from QM6a. The donor organism is *A. niger*. Alpha-glucosidase expression cassette was integrated into the host genome. Full details of the genetic modifications are provided in Appendix E (Confidential Commercial Information).

The genetic stability of the inserted gene has been demonstrated by Southern Blot. Broth samples were taken prior and after prolonged fermentation mimicking commercial fermentation conditions. Samples were then used for genomic DNA extraction, digestion and probed with alpha-glucosidase gene. No change in band pattern was observed between the genomic DNA samples extracted from shake flask culture before serial transfer culture and those extracted after 10 days of serial shake flask culture. The results demonstrate that the insertion cassette has been stably maintained through generations during the fermentation process.

Full details of the genetic modifications and stability of the inserted genes are provided in Appendix E. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

4. Dietary exposure

Refer to Appendix C for further details

4.1. List of food or food groups likely to contain the enzyme or its metabolites

According to the food group classification system used in Standard 1.3.1-Food Additives Schedule 15 (15-5), alpha-glucosidase will be used in production of:

- 14.2.5 Spirits and liqueurs
- 11.2 Sugars and sugar syrups

In addition, products made with alpha-glucosidase, e.g. IMO and MSG, lactic acid, lysine, are also used as food additives or food ingredients, and would subsequently be used in production of all food categories where these food additives or ingredients are allowed.

4.2. Levels of residues in food

The proposed application rate of Alpha-glucosidase in decomposition or hydrolysis reaction for production of is 2-4 kg product/ton of raw material for biochemicals, and 6 to 20 ppm added during fermentation for potable alcohol. The proposed application rate of Alpha-glucosidase in synthetic or transfer reaction for starch processing in IMO production is 0.5-1.5 kg product/ton of starch. DuPont IB expects Alpha-glucosidase to be inactivated or removed during the subsequent production and refining processes for all applications.

In decomposition or hydrolysis reaction, Alpha-glucosidase is added to the fermentation tank. The enzyme is then deactivated by low pH and/or high temperature during the manufacturing process. In addition, the deactivated enzyme is removed by the crystallization or other purification steps.

In synthetic or transfer reaction, Alpha-glucosidase performs its technological function after the saccharification step for production of IMO syrup by catalysis of the hydrolysis of starch. The Alpha-glucosidase is denatured by heat during a dedicated inactivation step or removed during subsequent carbon or ion exchange treatments.

The most appropriate way to estimate the human consumption in the case of food enzymes is using the Budget Method (Hansen, 1966; Douglass *et al.*, 1997). This method enables one to calculate a Theoretical Maximum Daily Intake (TMDI) based on conservative assumptions regarding physiological requirements for energy from food and the energy density of food rather than on food consumption survey data. The Budget Method was originally developed for determining food additive use limits and is known to result in conservative estimations of the daily intake.

Based on the raw materials used in the various food processes, the recommended use levels of the enzyme Alpha-glucosidase, for the calculation of the TMDI, the maximum use levels are chosen. The TMDI is calculated on basis of the maximal values found in food and beverages multiplied by the average consumption of food and beverages per kg body weight/day. Consequently, the TMDI will be: 0.443 mg TOS/kg body weight/day. The NOAEL has been determined for Alpha-glucosidase to be at 63.64 mg total protein/kg bw/day (equivalent to 77.2 mg TOS/kg bw/day). Based on a worst-case scenario of daily food consumption, the NOAEL would offer a 174X fold margin of safety. It should be stressed that this Total TMDI is based on

conservative assumptions and represents a highly exaggerated value. Please refer to Appendix C for details.

4.3. Percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid

The enzyme would be used as a processing aid in about:

- 100% of the tonnage of IMO syrup products sold in Australia and New Zealand
- 50% of the tonnage of MSG, lysine, other organic acid products, and potable alcohol sold in Australia and New Zealand

4.4. Levels of residues in food in other countries

Applications and levels of use of the Alpha-glucosidase preparation in other countries is the same as presented in section 4.2.

5. References

Bissett J (1984). A revision of the genus *Trichoderma*. I. Section *Longibrachiatum* sect. nov. Canadian Journal of Botany, 62(5), 924-931

Blumenthal CZ (2004). Production of toxic metabolites in *Aspergillus niger, Aspergillus oryzae, and Trichoderma reesei*: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. Regulatory Toxicology and Pharmacology, 39(2), 214-228

Brückner H, Graf H (1983). Paracelsin, a peptide antibiotic containing α-aminoisobutyric acid, isolated from *Trichoderma ressei* Simmons Part A.Experientia, 39(5), 528-530

JECFA (Joint FAO/WHO Expert Committee on Food Additives) 2006. General Specifications and Considerations for Enzyme Preparations Used in Food Processing.

Khuls K, Lieckfeldt E, Samuels GJ, Kovacs W, Meyer W, Petrini O, Gams W, Börner T, Kubicek CP (1996). Molecular evidence that the asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina*, Proc. Natl. Acad. Sci. USA 93, 7755-7760

Mandels M and Reese ET (1957). Induction of cellulase in Trichoderma viride as influenced by carbon sources and metals. J Bacteriol. 73, 269-278

Meyer W, Morawetz R, Borner T, Kubicek CP (1992). The use of DNA-fingerprint analysis in the classification of some species of the Trichoderma aggregate. Curr. Genet. 21, 27-30

Nevalainen H, Suominen P, Taimisto K (1994). On the safety of *Trichoderma reesei*, J. Biotechnol. 37, 193-200

Olempska-Beer ZS, Merker RI, Ditto MD, DiNovi MJ (2006). Food-processing enzymes from recombinant microorganisms—a review. Regul Toxicol Pharmacol 45, 144-158

Pariza,MW, Johnson EA (2001). Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing – Update for a New Century. Regul Toxicol Pharmacol, 33(2), 173-86,

Solfrizzo M, Altomare C, Visconti A, Bottalico A, Perrone G (1994). Detection of peptaibols and their hydrolysis products in cultures of *Trichoderma* species. Natural toxins, 2(6), 360