A Glucoamylase Enzyme
from a recombinant strain of *Tricoderma reesei*

PROCESSING AID APPLICATION

Food Standards Australia
New Zealand

Applicant: Danisco New Zealand Ltd

November 11, 2019

CONTENTS

EXECUTIVE SUMMARY:

DuPont Nutrition & Biosciences (N&B) is seeking approval for a "glucoamylase (EC 3.2.1.3)" enzyme for use as processing aid in bakery, brewing, potable alcohol, and starching processing. The enzyme is designated as "Glucoamylase" throughout the dossier.

The enzyme Glucoamylase is derived from a selected non-pathogenic, non-toxigenic strain of *Trichoderma reesei* which is genetically modified to overexpress the glucoamylase gene from *T. reesei.*

The enzyme is intended for use in baking, brewing, potable alcohol production and starch processing. In all these applications, glucoamylase convert starchy substrate to simple sugars, which can increase fermentation efficiency, and/or be converted to something sweeter in the case of starch processing. In all of these applications, Glucoamylase 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 Glucoamylase for use in these applications, Dupont N&B 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 Glucoamylase 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.

The safety of the food enzyme from *T. reesei* has been assessed using toxicology studies conducted on earlier strains of the DuPont *T. reesei* Safe Strain Lineage. The most suitable standard package of toxicological tests from the Safe Strain Lineage was identified to support the safety of the food enzyme object of the current dossier. The toxicological tests showed the following results:

- Ames test: no mutagenic activity under the given test conditions
- Chromosomal aberrations: no clastogenic activity under the given test conditions
- 90-day oral toxicity on rats: The No Observed Adverse Effect Level (NOAEL) is 1000 mg total organic solid (TOS)/kg bw/day (equivalent to 808 mg total protein/kg bw/day), which is the high dose in the study

Based on a conservative assumption and a highly exaggerated value consumption data, the NOAEL still offers a 314 fold Margin of Safety.

Based on the results of safety studies and other evidence, Glucoamylase 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 process of bakery, brewing, potable alcohol production and starch processing, lowering the manufacturing cost, and improving quality of final foods.

General information

1.1 Applicant details

(a) Applicant:

This application is made by Danisco New Zealand Ltd

(b) Company:

Dansico New Zealand Ltd

(c) Address:

[redacted]

(d) Contact Details:

[redacted]

(e) Email Address:

See above

(f) Nature of Applicants Business:

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

(g) Details of Other Individuals etc.:

[redacted]

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 is bakery, brewing, potable alcohol production and starch processing.

This application is made solely on behalf of DuPont Nutrition & Biosciences (N&B), 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.

Glucoamylase, subject of this application, is intended for use in baking, brewing, potable alcohol production and starch processing.

Currently no Glucoamylase from *Trichoderma reesei,* or from *T. reesei* expressed in *T. reesei* is permitted as a Processing Aid, however glucoamylase from *Aspergillus niger* and other microorganisms, and other enzymes including Cellulase, Endo-1,4-beta-Subtilisin, 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

Glucoamylase is an enzyme produced by submerged fermentation of *T. reesei* carrying the gene encoding the glucoamylase gene from *T. reesei.* The enzyme is characterized as a Glucan 1,4-alpha-glucosidase (EC 3.2.1.3). 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.

The enzyme is intended for use in in baking, brewing, potable alcohol production and starch processing. In all these applications, glucoamylase convert starchy substrate to simple sugars, which can increase fermentation efficiency, and/or be converted to something sweeter in the case of starch processing.

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 glucoamylase from *T. reesei*, or from *T. reesei* expressed in *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 glucoamylase from *T. reesei* 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 glucoamylase derived from *T. reesei* containing the gene for glucoamylase from *T. reesei* 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 N&B 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 Appendices B1, B3, -B6, Appendices D3, Appendices E1-E2, E4-E5, Partial of E3 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. In addition, all toxicological studies submitted to support this application is also considered **Confidential Commercial Information.** 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

Glucoamylase from *T. reesei* produced by *T. reesei* has been reviewed by JECFA. Please refer to Appendix D1 for JECFA specification of Glucoamylase from *T. reesei* produced by *T. reesei.*

1.8.2 International Legislation

Glucoamylase derived from *T. reesei* carrying the gene encoding the glucoamylase gene from *T. reesei* has been determined to be Generally Recognized as Safe (GRAS) in the United States as a food processing aid in production of bakery, brewing, potable alcohol production

and starch processing by a panel of scientific experts in the USA. It is also the subject of GRAS Notice 000372 with a concurrence letter received from FDA, dated Jul 25, 2011. It is also approved for various purposes in both France and Denmark. Refer Appendix D.

1.9. Statutory declaration

I, Caroline Elizabeth Gray,

of 5 Te Kare Rd, Wai O Taiki Bay, Aucklnad 1072, New Zealand, regulatory affairs manager:

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 [redacted]

Declared at Auckland on 11 of November 2019

Before me [redacted]

Signature: [redacted]

1.10. Checklist

	Mandatory Requirements	Check	Page Number	Remarks
General requirements for applications	A. Form of the application	√	N.A.	
	Table of contents	√	1	
	Executive summary	√	2	
	B. Applicant details	√	3	Section 1.1
	C. Purpose of application	√	4	Section 1.2
	D. Justification for the application	√	4	Section 1.3
	D.1 Regulatory impact information	√	4	Section 1.3.1
	D.1.1 Costs and benefits of the application	√	4	Section 1.3.1
	D.1.2 Impact on international trade	√	4	Section 1.3.1
	E Information to support the application	√	5	Section 1.4
	E.1 Data requirements	√	N.A.	
	F. Assessment procedure	√	5	Section 1.5
	G. Confidential commercial information (CCI)	√	5	Section 1.6
	H. Other confidential information	√	5	Section 1.6
	I. Exclusive capturable commercial benefit (ECCB)	√	5	Section 1.7
	J. International and other national standards	√	5	Section 1.8
	J.1 International Standards	√	5	Section 1.8.1
	J.2 Other national standards or regulations	√	5	Section 1.8.2
	K. Statutory declaration	√	7	Section 1.9
	L. Checklist	√	8	Section 1.10
3.3.2. Processing aids	A. Technical information on the processing aid	√	10	Section 2
	A.1 Information on the type of processing aid	√	10	Section 2.1
	A.2 Information on the identity of the processing aid	√	10	Section 2.2
	A.3 Information on the chemical and physical properties of the processing aid	√	10	Section 2.3
	A.4 Manufacturing process	√	12	Section 2.4
	A.5 Specification for identity and purity	√	12	Section 2.5
	A.6 Analytical method for detection	X		Not applicable for enzymes used as processing aids
	C. Information related to the safety of an enzyme processing aid	√	13	Section 3
	C.1 General information on the use of the enzyme as a food processing aid in other countries	√	13	Section 3.1

	C.2 Information on the potential toxicity of the enzyme processing aid	√	14	Section 3.2
	C.3 Information on the potential allergenicity of the enzyme processing aid	√	15	Section 3.3
	C.4 Safety assessment reports prepared by international agencies or other national government agencies, if available	√	15	Section 3.4
	D. Additional information related to the safety of an enzyme processing aid derived from a microorganism	√		Section 3.5-3.7
	D.1 Information on the source microorganism	√	15	Section 3.5
	D.2 Information on the pathogenicity and toxicity of the source microorganism	√	16	Section 3.6
	D.3 Information on the genetic stability of the source organism	√	16	Section 3.7
	E. Additional information related to the safety of an enzyme processing aid derived from a genetically-modified microorganism	√	16	Section 3.8
	E.1 Information on the methods used in the genetic modification of the source organism	√	16	Section 3.8
	F Information related to the dietary exposure to the processing aid	√	18	Section 4
	F.1. A list of foods or food groups likely to contain the processing aid or its metabolites	√	18	Section 4.1
	F.2 The levels of residues of the processing aid or its metabolites for each food or food group	√	18	Section 4.2
	F.3 For foods or food groups not currently listed in the most recent Australian or New Zealand National Nutrition Surveys (NNSs), information on the likely level of consumption	√	19	Section 4.3
	F.4 The percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid	√	19	Section 4.4
	F.5 Information relating to the levels of residues in foods in other countries	√	19	Section 4.5
	F.6 For foods where consumption has changed in recent years, information on likely current food consumption	√	19	Section 4.6

2. Technical information

Please refer to Appendix A for further details

2.1. Type of processing aid

The Glucoamylase enzyme is an enzyme produced by submerged fermentation of *T. reesei*, carrying the glucoamylase gene from *T. reesei*.

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:

According to IUBMB Enzyme Nomenclature, the systematic name of the principle enzyme activity is 4-α-D-glucan glucohydrolase. Other names used are glucan 1,4-α-glucosidase, amyloglucosidase, γ-amylase; lysosomal α-glucosidase; acid maltase; exo-1,4-α-glucosidase; glucose amylase; γ-1,4-glucan glucohydrolase; acid maltase; 1,4-α-D-glucan glucohydrolase.

- EC number: 3.2.1.3
- CAS number: 9032-08-0

Biological source: The glucoamylase enzyme is an enzyme produced by submerged fermentation of *T. reesei*, carrying the glucoamylase gene from *T. reesei*.

2.2.2 Marketing Name of the Processing Aid:

The marketing name of this enzyme preparation will depend on the application. An example marketing name of Glucoamylase is DIAZYME® TGA.

2.2.3 Molecular and Structural Formula:

Glucoamylase is a protein. The amino acid sequence is known. Please refer to Appendix E.

2.3. Chemical and physical properties

In principle, the enzymatic conversion of polysaccharides such as starch, amylose, amylopectin and dextrin with the help of Glucoamylase can be of benefit in the processing of food raw materials, which naturally contain the substrate.

The benefits of the use of Glucoamylase in certain food processes may include:

- Baking:
  - Reduce the need to add simple sugars to the formulation
  - Enable the making of no sugar added yeast-fermented breads
  - Lower the yeast usage as yeast vigor may be reduced by high sugar levels

- Brewing:
  - Maximized conversion of starchy substrates to fermentable carbohydrate
  - Potential for higher alcohol yield ○ Increased beer attenuation
  - Potential for use less raw material ○ Increased flexibility in the choice of raw materials

- Potable alcohol production:
  - Maximized conversion of starchy substrates to fermentable carbohydrate
  - Potential for higher alcohol yield
  - Potential for use less raw material
  - Increased flexibility in the choice of raw materials

- Starch processing:
  - Maximized conversion of liquefied starch to monomers, which can be converted enzymatic to something sweeter
  - Maximized conversion of liquefied starch to monomers/substrate, which can be further fermented to ethanol, citric acid, lactic acid and other food ingredients

Substrate specificity:

Glucan 1,4-alpha-glucosidase (IUBMB 3.2.1.3) hydrolyses terminal (1→4)-linked αD-glucose residues successively from non-reducing ends of polysaccharides with release of β-D-glucose. Substrates include polysaccharides such as starch, amylose, amylopectin, dextrin and glycogen.

Activity:

The activity of the Glucoamylase is defined in GAU. This assay is based on the ability of glucoamylase 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 glucoamylase activity and is monitored at 400nm via the use of an enzyme standard.

Temperature optimum:

Approximately between 63-73°C, with activities observed from 30 to nearly 90°C.

Thermal stability:

The enzyme activity dropped to below detection after incubation at 70°C for less than 10 minutes.

pH optimum:

Approximately pH 4.0-5.0, with high relative activity at pH interval 3.5-5.5.

pH stability:

Optimal stability is seen at the pH interval 4.0-5.0 and the enzyme activity is observed in the pH range 3.0-6.5.

Interaction of the enzyme with different foods:

The Glucoamylase 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:

Glucoamylase is a protein and any residual amounts remaining in food consumed would accordingly have the same nutritional value. However, the use levels of Glucoamylase 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 Glucoamylase 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 Glucoamylase 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
Arsenic	less than 3 mg/kg
Cadmium	less than 0.5 mg/kg
Mercury	less than 0.01 mg/kg
Microbiological:	
Total coliforms	less than 30 CFU/mL
E. coli	absent in 25 mL
Salmonella	absent in 25g mL
Antibiotic activity	Negative by test
Physical properties:	
Appearance	Tan to brown liquid

Standard for identity:

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

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 *Trichoderma reesei* glucoamylase (also known as glucan 1,4-α-glucosidase) shows a clear conserved Glyco_hydro_15 superfamily sequence domain characteristic for glucoamylase activities, together with a CBM20_glucoamylase family starch binding domain.

A selection of glucoamylase sequences of the species listed on Schedule 18 of the ANZ Food Standards Code were retrieved from the UniProtKB database and analysed for homology. The highest homology between the glucoamylase enzyme subject of this dossier and analyzed glucoamylase enzymes present on Schedule 18 of the ANZ Food Standards Code is 52.39% identity with the *A. oryzae* glucoamylase. The identity between the FSANZ approved glucoamylases (*A. niger*, *A. oryzae*, *R. delemar*, *R. oryzae*, *R. niveus*) ranges from 36.66% (*R.*

oryzae var. delemar glucoamylase amyB to *A. niger* glucoamylase glaA) to 92.80% (*R. oryzae* var. delemar glucoamylase amyB to *R. oryzae* var. delemar glucoamylase 1). Note that the UniProtKB database does not contain glucoamylase entries from *Rhizopus oryzae* itself, but only contains *Rhizopus delemar* and *Rhizopus oryzae* var. delemar glucoamylase entries. The UniProtKB database does not contain any *R. niveus* glucoamylase sequences. It is good to realize that the glucoamylase sequences within one species can show strain dependent amino acid sequence variability. Also, several microorganism species contain more than one glucoamylase encoding genes with different sequences (e.g. three *R. oryzae* var. delemar glucoamylase sequences).

3.2. Toxicity of the enzyme

Toxin homology study

A BLAST search for homology of the Glucoamylase sequence against the complete Uniprot database (http://www.uniprot.org/), was performed, with a threshold E-value of 0.1. The majority of matches were glucoamylases, 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 glucoamylase sequence was performed against the Uniprot animal toxin database. This yielded no matches.

Therefore, the Glucoamylase sequence does not share homology with a known toxin or venom sequence.

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.

Toxicological testing

A review of toxicology studies conducted with enzyme preparations produced by *T. reesi* strains indicates that, regardless of the *T. reesei* production strain, all enzyme preparations are not mutagenic, clastogenic or aneugenic in genotoxicity assays and do not adversely affect any specific target organ. Due to the consistency of the findings from enzyme preparations derived from different *T. reesei* strains, it is expected that any new enzyme preparation produced from *T. reesei* strains would have a similar toxicological profile.

DuPont N&B has determined by scientific procedures that production organism *T. reesei* TrGA-LOVMC2#3 is safe as a production organism as it pertains to the DuPont *T. reesei* Safe Strain

Lineage (see Appendix B) – more specifically the '*T. reesei* Host Strain #4 (M1-1.1)' branch. The position of the food enzyme object of the current dossier as well as the position of the strain providing the supportive toxicological studies is presented in the DuPont *T. reesei* Safe Strain Lineage (Appendix B).

For the safety assessment of Glucoamylase from *T. reesei* TrGA-LOVMC2#3, the data based on *T. reesei 'T. reesei* (heterol. rDNA) Trehalase strain' (Strain number 17 as in the SSL in Appendix B2 and Appendix B3) with a NOAEL of 1,000 mg TOS/kg bw/day is used as bridging data, because it is most closely related to TrGA-LOVMC2#3 based on strain lineage. The toxicology data from '*T. reesei* (heterol. rDNA) Trehalase strain' is also selected for the following reasons:

1. The 90-day oral (gavage) study was conducted according to OECD guideline 408 and in compliance with all current GLP regulations.
2. Genotoxicity studies (Bacterial reverse mutation assay and *in vitro* chromosomal aberration assay with human peripheral lymphocytes) are available for*'T. reesei* (heterol. rDNA) Trehalase strain'. The data show no evidence of genotoxicity.

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. Allergenicity of the enzyme

Bioinformatic analyses based on sequence homology determined that the *T. reesei* glucoamylase is unlikely to pose a risk of food allergenicity. Refer to Appendix B for additional information on the safety of the enzyme as to its allergenicity potential.

An allergen statement is given in Appendix A9.

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

As discussed in section 1.8, Glucoamylase from T. reesei produced by T. reesei has been reviewed by JECFA, determined to be GRAS in the United States, and approved in both France and Denmark for various purposes. Refer Appendix D for safety reports/approval letters.

3.5. Information on the source micro-organism

The production organism strain TrGA-LOVMC2#3 is a strain of *T. reesei* which has been genetically modified by DuPont N&B to overexpress a glucoamylase from *T. reesei*.

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). The Glucoamylase gene was placed under the expression signals of the endogenous *T. reesei cbh1* gene, and *T. reesei* cbh1 terminator.

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.

3.6. Pathogenicity and toxicity of the source micro-organism

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, 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. Further details are discussed in Appendix B.

3.7. Genetic stability of the source organism

The parental strain of the production strain *T.reesei* QM6a and its derivatives have been used for industry scale enzyme manufacturing for decades by DuPont N&B and its parental companies, and has demonstrated stable enzyme expression even at large scale fermentation. Please also refer to Appendix B2 for list of example enzyme preparations produced using *T.reesei* QM6a and its derivatives. Furthermore, the production strain has demonstrated to be 100% stable as confirmed by Southern Blot.

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 and Southern Blot analysis. No change in band pattern was observed between samples prior and after fermentation. The results demonstrate that the insertion cassettes have been stably maintained through generations during the fermentation process.

Refer Appendix E 3 for detailed information.

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

The production organism of the Glucoamylase preparation, the subject of this submission, is *T. reesei* strain TrGA-LOVMC2#3. It is derived by recombinant DNA methods from strain RL-P37. The purpose of this genetic modification is to enhance glucoamylase production levels. 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 *T.reesei*. Glucoamylase expression cassettes were integrated into the host genome. Full details of the genetic modifications are provided in Appendix E2 (Confidential Commercial Information).

Full details of the genetic modifications and stability of the inserted genes are provided in Appendix E1-E3. 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), Glucoamylase will be used in:

- 7. Bread and Bakery Products
- 14. Non-alcoholic and alcoholic beverages

In addition, products made with glucoamylase, e.g. sweeteners, 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 Glucoamylase in its intended application is listed below.

Application	Raw material (RM)	Recommended use levels (mg TOS/kg RM)	Maximal recommended use 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

DuPont N&B expects the Glucoamylase to be inactivated or removed during the subsequent production and refining processes for all applications.

Glucoamylase typically performs its technological function during the dough or batter handling, reducing or eliminating the need to add simple sugars to the formulation. Glucoamylase is denatured by heat during the baking or steaming step.

In brewing, Glucoamylase is typically added in mashing step and is therefore denatured in the consecutive lautering or mash filtration step. Glucoamylase may also be added during the fermentation step. In this case Glucoamylase will be denatured during the pasteurization step.

In potable alcohol production, Glucoamylase is added in the pre-treatment, liquefaction, pre-saccharification or the fermentation step. In Potable alcohol production, solids are separated from the fermentation slurry at the end of fermentation and any enzyme protein precipitate will be removed with the solids. The liquids are distilled. The distilled alcohol is subsequently filtered through a molecular sieve at temperatures well over boiling to adsorb further traces of water and water-soluble proteins. Therefore, the Glucoamylase will not be present/active in the end product due to distillation in the case of alcohol production.

In starch processing to produce corn sweeteners such as HFCS, the enzyme is added after the liquefaction step, to cooked starch. The syrup is then filtered and clarified by centrifugation, carbon treatment and ion exchange chromatography to remove any objectionable flavor or color. The purified hydrolysate passes a check filter and the clear hydrolysate is evaporated to reduce the amount of water. The various additional purification steps (filtration, carbon treatment, ion exchange), to which the glucose syrup is subjected, effectively remove all enzyme protein. Negligible carryover of the Glucoamylase is expected.

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 Subtilisin, 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: 3.18 mg TOS/kg body weight/day. The NOAEL has been determined for Glucoamylase to be at 1000 mg total protein/kg bw/day (equivalent to 1190.5 mg TOS/kg bw/day). Based on a worst-case scenario of daily food consumption, the NOAEL would offer a 374 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. Likely level of consumption of foods or food groups not currently listed in the most recent Australian or New Zealand National Nutrition Surveys (NNSs)

Not applicable. Glucoamylase is not expected to be used in production of any foods or food groups that are currently not listed in NNSs. If such usage arises, an application would be made to inform FSANZ.

4.4. 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:

- 20% of the tonnage of bread and bakery products sold in Australia and New Zealand
- 50% of Potable alcohol products
- 20% of Brewing products
- 25% of Sweeteners

4.5. Levels of residues in food in other countries

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

4.6. Likely current food consumption for foods where consumption has changed in recent years

Not applicable. Consumption of foods (including bakery good, alcoholic drinks, non-alcoholic drinks) produced with Glucoamylase is not expected to have a significant change.

5. References

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