Alpha-glucosidase Enzyme

Preparation Derived from

Trichoderma reesei

Expressing the Alpha-glucosidase Gene

from

Aspergillus niger

Is Generally Recognized As Safe

For Use in Food Processing

Notification Submitted by Danisco US Inc. (operating as DuPont Industrial Biosciences)

April 24, 2017

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1. GENERAL INTRODUCTION, STATEMENT AND CERTIFICATION

In accordance with 21 C.F.R. §170.225, Danisco US Inc. submits this GRAS Notice for α-glucosidase produced by *Trichoderma reesei* expressing the gene encoding the α-glucosidase enzyme from *Aspergillus niger*.

Genencor, a Danisco Division submitted a GRAS Notice on December 7, 2009 for a transglucosidase enzyme preparation from *Trichoderma reesei* expressing a transglucosidase from *Aspergillus niger,* which was intended for use as processing aid in the production of isomalto-oligosaccharide (IMO) syrups from starch and potable ethanol from molasses. FDA filed this notice on January 6, 2010, designated it as GRN 315, and issued a "No-Questions Letter" in response to the notice on May 5, 2010 (Appendix 1).

The *A. niger* enzyme in GRN 315 was identified historically by its main activity as transglucosidase (IUBMB # 2.4.1.24), which by definition catalyzes both the hydrolysis and transference of D-glucosyl units of oligosaccharides and the conversion of 1,4 glucosidic linkages to 1,6 glucosidic linkages. Indeed, when used in the production of specialty syrups from starch, this activity converts malto-oligosaccharides to isomalto-oligosaccharides (IMOs). Similarly, in molasses, transglucosidase converts non-fermentable sugars such as raffinose and stachyose to sucrose, galactose, glucose and fructose, which can then be fermented into alcohol.

In addition to transglucosidase activity, the same *A. niger* enzyme also has an α-glucosidase activity, which is the subject of this GRAS Notice. The α-glucosidase (IUBMB # 3.2.1.20, CAS # 9001-42-7) catalyzes the hydrolysis of terminal, non-reducing (1→4)-linked alpha-D-glucose residues with release of alpha-D-glucose. In fact, based on sequence identity, the gene encoding for the enzyme that displays both the transglucosidase and α-glucosidase activities is identified in Genbank as *aglA* (Genbank accession: D45356.1) and the scientific name for this enzyme is α-glucosidase (GenBank: BAA23616.1), even though functionally this enzyme also displays transglucosidase activity (Duan *et al,* 1995; Nakamura *et al*, 1997). FDA was notified of the correct scientific name of the gene and enzyme in a letter dated March 28, 2011, to which FDA responded on June 17, 2011, stating it preferred to maintain the identity of the substance as transglucosidase (Appendix 2).

The α-glucosidase enzyme preparation is intended for the hydrolysis of terminal, non-reducing (1→4)-linked alpha-D-glucose residues with release of alpha-D-glucose used in the fermentation to manufacture potable alcohol, organic acids (*e.g.,* lactic acid, citric acid and succinic acid), and monosodium glutamate (MSG). In these applications, α-glucosidase will be used as a processing aid and be either not be present in the final food or will be present in insignificant quantities as inactive residue, having no function or technical effect in the final food.

The systematic name of the principal enzyme activity is α-D-glucoside glucohydrolase. This enzyme is also known as acid maltase, glucoinvertase, *etc*., as described in Section 2.2.1 of this submission.

The enzyme hydrolyzes terminal, non-reducing (1→4)-linked α-D-glucose residues with the release of α-D-glucose.

The EC number of the enzyme is 3.2.1.20, and the CAS number is 9001-42-7.

The information provided in the following parts is the basis of our determination of GRAS status of this α-glucosidase enzyme preparation.

Our safety evaluation is in agreement with the recent publication by the Enzyme Technical Association (Sewalt *et. al.*, 2016, see Appendix 3), which includes an evaluation of the production strain, the enzyme, and the manufacturing process, as well as a determination of dietary exposure (see Part 3 of this submission).

The safety of the production organism must be the prime consideration in assessing the safety of an enzyme preparation intended for food use (Pariza & Johnson, 2001; Pariza & Foster, 1983). The safety of the production organism (*T. reesei* for the α-glucosidase) is discussed in Part 2 and 6 of this submission. Another essential aspect of the safety evaluation of enzymes derived from genetically engineered microorganisms is the identification and characterization of the inserted genetic material (Pariza & Johnson, 2001; Pariza & Foster, 1983; IFBC, 1990; EU Scientific Committee for Food, 1991; OECD, 1993; Berkowitz and Maryanski, 1989). The genetic modifications used to construct this production organism are well defined and are described in Part 2. The safety evaluation described in Part 3 and 6 shows no evidence to indicate that any of the cloned DNA sequences and incorporated DNA code for or express a harmful toxic substance.

1.1 Exemption from Pre-market Approval

Pursuant to the regulatory and scientific procedures established in proposed 21 C.F.R. §170.36, Danisco US Inc. has determined that its α-glucosidase enzyme preparation from a genetically engineered strain of *T. reesei* expressing the α-glucosidase enzyme from *Aspergillus niger* is a Generally Recognized As Safe ("GRAS") substance for the intended food applications and is, therefore, exempt from the requirement for pre-market approval.

1.2 Proposed § 170.36 (c)(l)(i) Name and Address of Notifier

Danisco US Inc.
(operating as DuPont Industrial Biosciences)
925 Page Mill Road
Palo Alto, CA 94304

1.3 Proposed § 170.36 (c)(l)(ii) Common or Usual Name of Substance

The α-glucosidase enzyme preparation is produced in a *Trichoderma reesei* strain expressing the gene encoding an α-glucosidase from *Aspergillus niger.*

1.4 Proposed § 170.36 (c)(l)(iii) Applicable Conditions of Use

The α-glucosidase is used as a processing aid during the fermentation to manufacture potable alcohol and organic acids (*e.g.,* lactic acid, citric acid and succinic acid) and monosodium glutamate (MSG) at 4 kg product/MT substrate (equivalent to 235 mg TOS/kg substrate).

1.5 Proposed §170.36 (c)(l)(iv) Basis for GRAS Determination

This GRAS determination is based upon scientific procedures in accordance with 21 C.F.R. §§170.30 (a) and (b).

1.6 Proposed § 170.36 (c)(l)(v) Availability of Information for FDA Review

A notification package providing a summary of the information that supports this GRAS determination is enclosed with this notice. The package includes a safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure. The complete data and information that are the basis for this GRAS determination are available for review and copying at 925 Page Mill Road, Palo Alto, CA 94304 during normal business hours or will be sent to the Food and Drug Administration upon request.

1.7 Disclosure and Certification

This GRAS notice does not contain any data and or information that is exempt from disclosure under the Freedom of Information Act (FOIA; 5 U.S.C §552).

We confirm that the data and information in this GRAS notice satisfactorily addresses Parts 2-7 of a GRAS notice per 21 C.F.R. §§170.230 to 170.255 as copied below:

170.230 Part 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect.
170.235 Part 3 of a GRAS notice: Dietary exposure.
170.240 Part 4 of a GRAS notice: Self-limiting levels of use.
170.245 Part 5 of a GRAS notice: Experience based on common use in food before 1958.
170.250 Part 6 of a GRAS notice: Narrative.
170.255 Part 7 of a GRAS notice: List of supporting data and information in your GRAS notice.

Danisco US Inc. certifies that to the best of our knowledge this GRAS notice is complete, representative, and a balanced submission that includes unfavorable and favorable information known to us as well as relevant to the evaluation of the safety and GRAS status of the use of the notified substance.

(b) (6)

April 24, 2017

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2. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATION AND PHYSICAL OR TECHNICAL EFFECT

2.1 PRODUCTION MICROORGANISM

2.1.1 Production Strain

The production organism is a strain of *Trichoderma reesei*, which has been genetically engineered through the deletion of several endogenous cellulase genes and the expression of a gene for the production and secretion of α-glucosidase from *A. niger*. This production strain is the same as the production strain described in GRN 315 for the transglucosidase, in which the enzyme has both transglucosidase and α-glucosidase activities. *T. reesei* is classified as a 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, and is also considered as part of Good Industrial Large Scale Practice (GILSP) worldwide. It also meets the criteria for a safe production microorganism as described by Pariza and Foster (1983). It contains the α-glucosidase gene under the regulation of a native *T. reesei cbh1* (cellobiohydrolase 1) gene. The *A. nidulans amdS* gene was used as a selectable marker. The inserted DNA was integrated into the recipient chromosome.

2.1.2 Host Microorganism

The host organism *T. reesei* strain RL-P37 was obtained from Dr. Montenecourt. The derivation and characterization of strain RL-P37 has been published by Sheir-Neiss and Montenecourt (1984). Strain RL-P37 is a cellulase over-producing strain that was obtained through several classical mutagenesis steps from the wild-type *Trichoderma reesei* strain (QM6a). Strain QM6a is present in several public culture collections, *e.g.,* in the American Type Culture Collection as ATCC 13631. *T. reesei* has more recently been identified as a clonal derivative or anamorph of *Hypocrea jecorina* (Dugan, 1998 and Khuls *et al*., 1996).

2.1.3 Alpha-glucosidase Expression Vector

As previously described in GRN 315, for construction of the vector, the DNA encoding the *A. niger* mature secreted α-glucosidase, which also has transglucosidase activity, was fused to the *T. reesei* CBH1 signal peptide. This open reading frame is flanked by the promoter and terminator sequences of the *T. reesei cbh1* gene. The vector also contains the *Aspergillus nidulans amdS* gene, encoding acetamidase, which is a common selectable marker for transformation of *T. reesei*.

The genetic construction was evaluated at every step to assess the incorporation of the desired functional genetic information, and the final construct was verified by Southern blot analysis to confirm that only the intended genetic modifications to the *T. reesei* strain had been made.

2.1.4 Stability of the Introduced Genetic Sequences

The production strain proved to be 100% stable after at least 60 generations of fermentation, as evaluated by α-glucosidase production.

2.1.5 Antibiotic Resistance Gene

No antibiotic resistance genes were used in the construction of the production microorganism, and therefore the final production strain does not contain any antibiotic resistance genes.

2.1.6 Absence of Production Microorganism in Product

The absence of the production microorganism in the final product is an established specification for the commercial product and utilizes an analytical method with a detection limit of 1 CFU/g. The production organism does not end up in the finish food and therefore, the first step in the safety assessment as described by the International Food Biotechnology Council (IFBC) is satisfactorily addressed.

2.2 ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

2.2.1 Enzyme Identity

IUBMB Nomenclature:	α-glucosidase
IUBMB Number:	3.2.1.20
CAS Number:	9001-42-7
Reaction catalyzed:	Hydrolysis of terminal, non-reducing (1→4)-linked alpha-D-glucose residues with release of alpha-D-glucose
Other Names:	maltase; glucoinvertase; glucosidosucrase; maltase-glucoamylase; α-glucopyranosidase; glucosidoinvertase; α-D-glucosidase; α-glucoside hydrolase; α-1,4-glucosidase
Systematic Name:	Alpha-D-Glucoside glucohydrolase

2.2.2 Amino Acid Sequence

The amino acid sequence of the α-glucosidase enzyme and nucleotide sequence of the gene encoding it is freely available from public databases such as GenBank (Accession numbers BAA23616, D45356). The sequence corresponding to the mature α-glucosidase (between the positions 1251 and 1252) in the GenBank sequence D45356 (Nakamura *et al*., 1997 and Kimura *et al*., 1992) was used for this construction.

The amino acid sequence of the α-glucosidase enzyme is shown in Appendix 4.

2.3 MANUFACTURING PROCESS

This section describes the manufacturing process for this α-glucosidase enzyme which follows standard industry practice (Kroschwits, 1994; Aunstrup *et al.*, 1979; Aunstrup 1979). For a diagram of the manufacturing process, see Appendix 5. The quality management system used in the manufacturing process complies with the requirements of ISO 9001. The enzyme preparation is also manufactured in accordance with FDA's current Good Manufacturing Practices ("cGMP") as set forth in 21 C.F.R. Part 110.

2.3.1 Raw Materials

The raw materials used in the fermentation and recovery process for this α-glucosidase concentrate (with transglucosidase activity in GRN 315) are standard ingredients used in the enzyme industry (Kroschwits 1994; Aunstrup 1979 and Aunstrup *et al.*, 1979). All the raw materials conform to the specifications of the Food Chemicals Codex, 10th edition, 2016 ("FCC"), except for those raw materials that do not appear in the FCC. For those not appearing in the FCC, internal requirements have been made in line with FCC requirements and acceptability of use for food enzyme production. Danisco US Inc. uses a supplier quality program to qualify and approve suppliers. Raw materials are purchased only from approved suppliers and are verified upon receipt.

The antifoam used in the fermentation and recovery is used in accordance with cGMP per the FDA correspondence to ETA acknowledging the listed antifoams dated September 11, 2003. The maximum use level of the antifoam in the production process is $\leq$0.15%.

Glucose (which may be derived from wheat) is used in the fermentation process and will be consumed by the microorganism as nutrients. The final enzyme preparation does not contain any major food allergens from the fermentation medium. No other major allergen substances are used in the fermentation, recovery processes, or formulation of this product.

2.3.2 Fermentation Process

The α-glucosidase enzyme is manufactured by submerged fermentation of a pure culture of the genetically engineered strain of *T. reesei* described in Part 2. All equipment is carefully designed, constructed, operated, cleaned and maintained to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are conducted periodically to ensure absence of foreign microorganisms and confirm production strain identity.

2.3.2.1 Production organism

A new lyophilized stock culture vial of the *T. reesei* production organism as described in Part 2 is used to initiate the production of each batch. Each new batch of the stock culture is thoroughly

controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

2.3.2.2 Criteria for the rejection of fermentation batches

Growth characteristics during fermentation are observed microscopically. Samples are taken from each fermentation stage (inoculum, seed, and main fermentor) before inoculation, at regular intervals during growth, and before harvest or transfer. These samples are tested for microbiological contamination by plating on a nutrient medium.

A fermentation batch is declared as 'contaminated' if colony forming units (CFU) of bacteria or fungi other than the production strain are present at levels $>10^3$CFUs/ml.

If a fermentation batch is determined to be contaminated, it will be rejected if deemed necessary. If the contamination is minor and determined to be from common non-pathogenic environmental microbes, the fermentation may be processed.

2.3.3 Recovery Process

The recovery process is a multi-step operation, which starts immediately after the fermentation process.

The enzyme is recovered from the culture broth by the following series of operations:

1. Primary separation –centrifugation or filtration;
2. Concentration – ultrafiltration;
3. Addition of stabilizers/preservatives; and
4. Polish filtration.

2.3.4 Formulation and standardization process

The ultra-filtered concentrate is stabilized by final formulation to contain 4.1 % dextrose and 0.4% sodium benzoate at pH 4.5-5.0. The remaining portion of the formulation is water.

The final α-glucosidase liquid concentrate from *T. reesei* is analyzed in accordance with the general specifications for enzyme preparations used in food processing as established by the Joint FAO/WHO Expert Committee on Food Additives ("JEFCA") in 2006 and Food Chemicals Codex, 10th edition, 2016 ("FCC"). These specifications are set forth in Section 2.4.

2.4 COMPOSITION AND SPECIFICATIONS

2.4.1 Quantitative Composition

The liquid concentrate is stabilized with formulation ingredients listed below and tested to demonstrate that it meets the specification.

Various commercial formulations exist, with a range of enzyme activities. The following is a representative composition:

- Enzyme activity 1650-2140 U/g
- Enzyme protein 10-15% (w/w)
- Sodium chloride 0.4% (w/w)
- Dextrose 4.1% (w/w)
- Water 80.5-85.5% (w/w)
- pH 4.5-5.0

2.4.2 Specifications

The α-glucosidase meets the purity specifications for enzyme preparations set forth in Food Chemicals Codex (FCC) 10th edition (US Pharmacopeia, 2016). In addition, it also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in Compendium of Food Additive Specifications (JECFA, 2006).

The results of analytical testing of the 3 lots of product is given in Appendix 6 verifying that the product meets the FCC 10th edition (2016) and JECFA (2006) specifications for enzyme preparations.

2.5 APPLICATION

2.5.1 Mode of Action

The α-glucosidase catalyzes the hydrolysis of terminal, non-reducing (1→4)-linked alpha-D-glucose residues with release of alpha-D-glucose.

2.5.2 Use Levels

The α-glucosidase preparation produced by strain *T. reesei* is intended for use in the fermentation manufacture of potable alcohol, organic acids (*e.g.,* lactic acid, citric acid and succinic acid), and monosodium glutamate (MSG). The product contains 5.875% TOS.

	Maximum Use Rate (kg Product/MT Substance)	**Resulting Exposure** (mg TOS/kg Substance)
Potable alcohol	4	235
Organic acids (e.g., Lactic acid, Citric acid)	4	235
MSG	4	235

2.5.3 Enzyme Residues in the Final Foods

As discussed above, the enzyme will be deactivated in both applications and will not be present in the final food products.

3. DIETARY EXPOSURE

Alpha-glucosidase is used as a processing aid in the fermentation to:

- Manufacture of potable alcohol;
- Manufacture of organic acid (*e.g.,* lactic acid, citric acid, and succinic acid); and
- Manufacture MSG.

While we expect no α-glucosidase to remain in the potable alcohol, organic acids, and MSG, the following calculations assume that 100% of the enzyme remains in the processed food.

As the enzyme protein contains two enzyme activities (α-glucosidase, the subject of this notice as well as transglucosidase, the subject of GRN 315), with two product lines, the following exposure assessment will include both the applications for α-glucosidase and transglucosidase as a consumer may be exposed to food processed with both enzyme activities.

Transglucosidase/α-glucosidase is used in grain/starch processing for production of fermentation products (potable alcohol, organic acids, and MSG) and isomalto-oligosaccharide (IMO) syrups. The dose rate and process yield from starch for alcohol, organic acids and MSG are set to be the same; the exposure to transglucosidase/α-glucosidase via potable alcohol from starch and molasses, organic acids, MSG, and IMO syrups is outlined here and below via the Budget Method. The estimated yield of the alcohol, organic acids, and MSG is 35% and for IMO syrups, 100%. Therefore, the concentration of TOS from transglucosidase/α-glucosidase in the fermentation products and IMO syrups can be calculated and it is summarized in the table below.

	Fermentables (potable alcohol, organic acid, and MSG)	Potable alcohol from molasses	IMO syrups
Dose (kg product/MT starch)	4	0.02	1.5
Dose (mg protein/kg starch)	200	1	75
Dose (mg TOS/ kg starch)	235	1.175	88.2
Yield %	35	0.25	100
Concentration (mg TOS/kg)	671.4	4.7	88.2

Liquid Foods

The worst-case concentration of TOS from fermentables in potable alcohol and ingredients used in liquid foods was derived as 671.4 mg TOS/L. However, the distillation process in potable

alcohol manufacture denatures and precipitates all enzyme protein and removes the vast majority of water-soluble substances from the alcohol, hence the actual concentration of TOS from α-glucosidase preparation in potable alcohol is negligible.

The concentration of organic acids in soft drinks is set at 0.13%. Soft drinks typically contain 5-6.5% w/w IMO syrup, which results in an average 57.5 g IMO syrup per L. The maximum inclusion rate for MSG in soup can be up to 1%. Therefore, a final concentration of TOS from transglucosidase/α-glucosidase in beverages and soup can be calculated as shown in the table below.

	Fermentation products (beverage)	IMO Syrups (beverage)	MSG (soup)
Maximum Concentration (mg TOS /L)	671.4	88.2	671.4
Ingredient concentration in liquid food (%)	0.13	5.75	1
Enzyme TOS concentration in in liquid food (mg TOS /L)	0.873	5.067	6.714
Total TOS Concentration in liquid food (mg TOS/L)	5.94		6.71

For selecting an overall maximum exposure via liquids, the worst-case TOS concentration as MSG in soup (6.71 mg TOS/L) is appropriate, because:

- In distilled spirits, the actual TOS concentration will be minimal compared to the maximum theoretical TOS concentration, as the enzyme protein and other organic solids will be removed in the distillation step;
- The maximum intake of alcohol will largely limit the maximum intake of any alcoholic drink the body can tolerate, not by the volume of the drink; and
- The enzyme exposure in soup via MSG is higher than the enzyme exposure in beverage via fermentables and IMO altogether.

Hence, the exposures from MSG were used in our risk assessment to represent worst case scenario exposures via intake of liquids, with the assumption that 25% of all consumed beverages/soups are manufactured from raw materials treated with the transglucosidase/α-glucosidase.

Solid Foods

This transglucosidase/α-glucosidase is used in grain/starch processing in the manufacture of IMO syrup which will then be used in cereal and confectionery. The organic acid fermented product will be used in bakery and dairy, and the MSG will then be used in the soup powder.

Taking into account that the maximum application rates of 200 mg protein/kg starch (235 mg TOS/kg starch) and the estimated yield of organic acids and MSG, 35%; the concentration of fermentation products in solid foods was derived as 671.4 TOS mg/L.

The estimated yield of starch converted into syrup is 1 kg starch/1 kg IMO. The most considerable applications are cereal and confectionery products with a maximum IMO content of 25% besides the less voluminous application area of confectionary (up to 97% IMO in hard candy); and in organic acids containing products such as bakery and dairy with a typical content of up to 2%.

Therefore, a final concentration of TOS from transglucosidase/α-glucosidase in solid food can be calculated as shown in the tables below, with the highest concentration in IMO.

	Fermentables (organic acids)	**IMO**	**MSG (powdered soup)**
Maximum Concentration (TOS mg/kg ingredient)	671.4	88.2	671.4
Ingredient concentrate in solid food (%)	2	25	1
Enzyme TOS concentration in solid food (TOS mg/kg solid food)	13.43	22.03	6.71

HUMAN EXPOSURE ASSESSMENT

Based on application rate, knowledge of process parameters, and logical consumption patterns, the resulting theoretical exposure to transglucosidase/alpha-glucosidase via liquid foods is highest from its use in MSG, which will be used in this risk assessment to represent a worst-case scenario. In this assessment, the highest concentration of 6.71 mg TOS/ L is used to represent a worst-case scenario for liquid foods. The concentration of transglucosidase/α-glucosidase in IMO for use in solid foods is 22.03 mg TOS/ kg food.

In this assessment, the Budget method is used. This method was previously used by JECFA (FAO/WHO, 2001) and uses the following assumptions:

1. Level of consumption of foods and beverages:

For solid foods, the daily intake is set at 25 g/kg bw based on a maximum lifetime energy intake of 50 Kcal/kg bw/day. For non-milk beverages, a daily consumption of 100 ml/kg bw is used corresponding to 6 liters per day for a 60-kg adult.

2. Concentration of enzymes in foods and beverages

The concentration of enzyme in foods and beverages is the maximum application rate.

3. Proportion of foods and beverages that contain the enzymes

a. A default of 50% of all solid foods is used to represent processed foods (*i.e.*, 12.5 g/kg bw/day).
b. A default of 25% is used to represent non-milk beverages that may contain the enzyme (*i.e.*, 25 ml/kg bw/day).

4. Estimation of the theoretical maximum daily intake (TMDI)

To represent a worst-case scenario, TMDI for solid foods must be combined with the TMDI for beverages in the risk assessment.

Estimation of the TMDI for Liquid Foods:

TOS mg/kg liquid food (soup)	6.71
TOS mg/25 mL (soup) via MSG	0.168

In this assessment, the TMDI for liquid foods is 0.168 mg TOS /kg BW/day.

Estimation of the TMDI for solid foods:

TOS mg/kg food	22.03
TOS mg/12.5 g food via IMO syrups	0.275

In this assessment, the TMDI for solid foods is 0.275 mg TOS/kg BW/day.

TMDI Total:

TMDI – Liquid Foods + TMDI – Solid Foods =
0.443 mg/TOS kg bw

4. SELF-LIMITING LEVELS OF USE

As the enzyme will be used as processing aid in the food manufacturing process, there is no notable oral intake for humans. Therefore, self-limiting levels of use are not applicable.

In addition, as a processing aid the self-limited levels of use are primarily economical as customers are unlikely use more enzyme than is needed to achieve the technical effects to minimize production costs.

5. EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

Information regarding this enzyme's common use in food before 1958 is not provided as the statutory conclusion of our GRAS status is based on scientific procedures and not common use before 1958.

6. SAFETY EVALUATION

6.1 SAFETY OF THE PRODUCTION STRAIN

The safety of the production organism must be the prime consideration in assessing the safety of an enzyme preparation intended for use in food (Pariza and Foster, 1983). If the organism is non-toxigenic and non-pathogenic, then it is assumed that foods or food ingredients produced from the organism, using current Good Manufacturing Practices, are safe to consume (IFBC, 1990). Pariza and Foster (1983) define a non-toxigenic organism as 'one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure' and a non-pathogenic organism as 'one that is very unlikely to produce disease under ordinary circumstances. *T. reesei* strains used in enzyme manufacture meet these criteria for non-toxigenicity and non-pathogenicity.

6.1.1 Safety of the host

T. reesei was first isolated from nature in 1944. The original isolate, QM 6a, 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 by Bissett (1984) that *T. reesei* be placed into synonymy with *T. longibrachiatum*. Later however, evidence emerged indicating that the two species are not identical (Meyer *et al.,* 1992; Dugan, 1998, see Appendix 7). The proposal by Khuls *et al.* (1996) that *T. reesei* was a clonal derivative of *Hypocrea jecorina* is being generally accepted in the scientific community, and the US National Center for Biotechnology Information (NCBI) refers to *T. reesei* as the anamorph of *H. jecorina*. Therefore, the names *Trichoderma reesei* and *Hypocrea jecorina* are in use in the scientific literature to refer to essentially the same microorganism species (Samuels *et al*, 2012, see Appendix 7). Unfortunately, the name *Trichoderma longibrachiatum* is also still used in various regulations (including 21 C.F.R §184.1250) and various enzyme positive lists around the globe, and continued use of this name as a synonym for *T. reesei* has begun to result in questions from regulators as *T. longibrachiatum* is increasingly associated with infection of immune-compromised individuals. The U.S. EPA's risk assessment on *T. reesei* (Federal Register / Vol. 77, No. 172 / September 5, 2012 / pages 54499-54411) stresses that it is not the species associated with infection of immune-compromised individuals, but rather this is *T. longibrachiatum*, hence the continued use on various national and international regulatory positive lists of *T. longibrachiatum* rather than *T. reesei* as an approved / acceptable enzyme production host needs to be revisited.

A review of the literature search on the organism (1972 - 2017) uncovered no reports that implicate *T. reesei* in any way with a disease situation, intoxication, or allergenicity among healthy adult humans and animals. The species is not present on the list of pathogens used by the EU (Directive Council Directive 90/679/EEC, as amended) and major culture collections worldwide. It is classified as a 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.

Brückner and Graf (1983) reported the isolation from *T. reesei* strain QM 9414 a peptaibol compound (*e.g.,* paracelsin) that exhibited antibiotic activity. 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 and commercially irrelevant and the growth conditions under which the compounds were produced are very different from those in standard enzyme manufacturing. The US EPA published a risk assessment (EPA, 2012) to support tiered exemption status for *T. reesei* QM 6A and its derivatives (including QM 9414), in which the Agency acknowledged that under normal submerged fermentation conditions paracelsin is not produced. Strain QM 9414 and its derivatives have been safe producers of commercial cellulase enzyme preparations for food applications. The enzyme manufacturers still confirm the industrial enzyme preparations do not to have antibiotic activity per the specifications recommended by the 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), Blumenthal (2004), and Olemska-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 considered a safe production organism and is the source organism of a range of enzyme products that are used as processing aids in the international food and feed industries. It is listed as a safe production organism for cellulases in the Pariza and Johnson paper (2001) and in Olempska-Beer *et al.* (2006). Various strains have been approved to produce commercial enzyme products internationally, for example, in Canada (Food and Drugs Act Division 16, Table V, Food Additives That May Be Used As Enzymes), the United States (21 C.F.R. §184.1250), Mexico, Brazil, France, Denmark, Australia/New Zealand, China, and Japan. To date, 13 enzymes produced in *T. reesei* have been notified to FDA/CFSAN as GRAS for their intended uses and received a "No questions" letter.[1]

The production organism of the α-glucosidase enzyme preparation, the subject of this submission, is *T. reesei* strain TG #626, which was produced from strain RL-P37 using recombinant DNA methods. The purpose of this genetic modification is to express the α-glucosidase from *A. niger* in *T. reesei*. RL-P37, a commercial production strain, is derived, as a result of several classical mutagenesis steps, from the well-known wild-type strain QM 6a. Virtually all *T. reesei* strains used all over the world for industrial cellulase production today are derived from QM 6a. DuPont Industrial Biosciences (formerly Genencor, a Danisco Division) has used strain RL-P37 to produce cellulases for over fifteen years and has developed many production strains from it using recombinant DNA techniques. The strain has been determined to be non-pathogenic and non-toxicogenic through an acute intraperitoneal study in rats. All the food/feed grade products produced by this lineage were determined to be safe for their intended uses and are the subject of numerous GRAS determinations. Five GRAS Notices were filed for the products from this strain lineage, in which FDA issued "no questions" letters (see GRN 230, GRN 315, GRN 333, GRN 372, and GRN 567).

[1] http://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&sort=GRN_No&order=DESC&startrow=1&type=basic&search=reesei

From the information reviewed, it is concluded that the organism *T. reesei* strain provides no specific risks to human health and is safe to use as the production organism of α-glucosidase. The strain is non-pathogenic and non-toxigenic.

6.1.2 Safety of the donor source

Aspergillus niger also has a long history of safe use in the production of industrial enzymes and chemicals of both food grade and technical grade. It is one of the most important producers of industrial neutral proteases (Uhlig 1998) and glucoamylases (Kroschwitz 1994). The species is listed as a production/donor organism for a series of food-grade carbohydrases, oxidoreductases, lipases, glucanotransferase, and proteases in the Pariza and Johnson paper (2001). Several *A. niger*-derived substances used in the food industry have acquired GRAS status for the proposed applications:

- Carbohydrase and cellulase enzyme preparation (21 C.F.R. §173.120);
- Citric acid (21 C.F.R. §173.280);
- Carbohydrase enzyme preparation, catalase enzyme preparation, glucose oxidase enzyme preparation, pectinase enzyme preparation, and protease enzyme preparation (GRN 89); and
- Lactase (GRN 132); Lipase (GRN 111 and GRN 296), Asparaginase (GRN 214 and GRN 428), Phosplipase A2 (GRN 183), Carboxypeptidase (GRN 345), Peroxidase (GRN 402), and Xylanase (GRN 589).

The safety of *A. niger* was summarized by Schuster *et al.* (2002) and was also discussed and supported by Olempska-Beer *et al.* (2006).

In 1997, *A. niger* became one of the ten microbial species/strains that were eligible for Tier 1 exemption under 40 C.F.R. § 725.420 as recipient microorganisms under the TSCA biotechnology regulations (1997), based on EPA's risk assessments.

The donor organism for the α-glucosidase gene used in construction of the new microorganism *T. reesei* TG #626 was *Aspergillus niger* AGME 9, also known as SE4, and described in US patent 5,783,414. *A. niger* AGME9 is the source of DuPont Industrial Biosciences' (formerly Genencor, a Danisco division) current α-glucosidase product. The enzyme from *A. niger* is one of the well-known forms of α-glucosidases (Yoshikawa *et al.,* 1994). The amino acid sequence of the enzyme and nucleotide sequence of the gene encoding it are freely available from public databases such as GenBank (Accession numbers BAA23616, D45356). The sequence corresponding to the mature α-glucosidase (between the positions 1251 and 1252 in the GenBank sequence D45356 (Nakamura *et al.*, 1997 and Kimura *et al.*, 1992) was used for this construction.

The *A. niger* α-glucosidase is GRAS as previously determined by Genencor and concurred by Dr. Michael Pariza. Meanwhile, the same enzyme protein with transglucosidase activity, the subject of GRAS Notice GRN 315 is GRAS determined and concurred by Dr. Michael Pariza as well. Additionally, it is described as the enzyme used to produce isomalto-oligosaccharides in GRAS Notice 246 and the corresponding FDA "no-questions" letter.

6.2 SAFETY OF THE MANUFACTURING PROCESS

The manufacturing process to produce α-glucosidase will be conducted in a manner similar to other food and feed production processes. It consists of a pure-culture fermentation process, cell separation, concentration, and formulation. The process is conducted in accordance with current food good manufacturing practice (cGMP) as set forth in 21 C.F.R. Part 110. The resultant product meets the purity specifications for enzyme preparations of the Food Chemicals Codex, 10th Edition (US Pharmacopeia, 2016) and the general specifications for enzyme preparations used in food processing proposed by WHO/JECFA (2006).

The fermentation process may utilize a wheat derived source of glucose that may contain trace amount of protein. This feedstock will be consumed by *T. reesei* as nutrients. The final enzyme preparation does not contain any major food allergens from the fermentation medium.

6.3 SAFETY OF ALPHA-GLUCOSIDASE

6.3.1 Allergenicity

According to Pariza and Foster (1983), there have been no confirmed reports of allergies in consumers caused by enzymes used in food processing. Alpha-glucosidase has been used in food processes to generate IMOs for several years and have generated no known safety concerns.

In 1998 the Association of Manufacturers of Fermentation Enzyme Products (AMFEP) Working Group on Consumer Allergy Risk from Enzyme Residues in Food reported on an in-depth analysis of the allergenicity of enzyme products. They concluded that there are no scientific indications that small amounts of enzymes in bread and other foods can sensitize or induce allergy reactions in consumers, and that enzyme residues in bread and other foods do not represent any unacceptable risk to consumers. Further, in a recent investigation of possible oral allergenicity of 19 commercial enzymes used in the food industry, there were no findings of clinical relevance even in individuals with inhalation allergies to the same enzymes, and the authors concluded "that ingestion of food enzymes in general is not considered to be a concern with regard to food allergy" (Bindslev-Jensen *et al.*, 2006).

Despite this lack of general concern, the potential that α-glucosidase could be a food allergen was assessed by comparing the amino acid sequence with sequences of known allergens in a public database, which is described in more detail below. Based on the sequence homology alone, it was concluded that the *A. niger* α-glucosidase is unlikely to pose a risk of food allergenicity.

The most current allergenicity assessment guidelines developed by the Codex Commission (2009) and Ladics *et al.* (2011) recommend the use of FASTA or BLASTP search for matches of 35% identity or more over 80 amino acids of a subject protein and a known allergen. Ladics *et al.* (2011) further discussed the use of the "E-score or E-value in BLAST algorithm that reflects the measure of relatedness among protein sequences and can help separate the potential random occurrence of aligned sequences from those alignments that may share structurally relevant similarities." High E-scores are indicative that any alignments do not represent biologically

relevant similarity, whereas low E-scores ($<10^{-7}$) may suggest a biologically relevant similarity (*i.e.*, in the context of allergy, potential cross reactivity). They suggest that the E-score may be used in addition to percent identity (such as > 35% over 80 amino acids) to improve the selection of biologically relevant matches. The past practice of conducting an analysis to identify short, six to eight, contiguous identical amino acid matches is associated with false positive results and is no longer considered a scientifically defensible practice.

The Codex Commission states:

> "A negative sequence homology result indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known allergens."

Aspergillus niger α-glucosidase (mature) sequence is given below in Appendix 4.

The search for 80-amino acid stretches within the sequence with greater than 35% identity to known allergens using the Food Allergy Research and Resource Program (FARRP) AllergenOnline database[2] January 18, 2017 V17, containing 2035 peer-reviewed allergen sequences[3] revealed no match (using E-value <0.1 as the cut-off) to sequences in the data base using the full sequence search capabilities. FASTA alignment of the above sequence also using the AllergenOnline database[4] revealed no additional matches (using E-value <0.1 as the cut-off) to sequences in the database using the full sequence search capabilities.

Although cautioned against in Codex (2009), researched by Herman *et al.* (2009) and further elaborated by Ladics *et al.* (2011) and on AllergenOnline.org there is no evidence that a short contiguous amino acid match will identify a protein that is likely to be cross-reactive and could be missed by the conservative 80 amino acid match (35%). This database does allow for isolated identity matches of 8 contiguous amino acids to satisfy demands by some regulatory authorities for this precautionary search. Performing the 8 contiguous amino acids search produced no sequence matches with known allergens.

Microbial enzymes acting environmental allergens have yet to be conclusively demonstrated to be active via the oral route. This concept was evaluated extensively in a recently published study (Bindslev-Jensen *et al.*, 2006) that failed to indicate positive reactions to 19 orally challenged commercial enzymes in a double-blind placebo controlled food challenge study with subjects with positive skin prick tests for the same allergens. The authors concluded that positive skin prick test results are of no clinical relevance to food allergenicity, and that ingestion of food enzymes in general is not a food allergen concern.

In conclusion, based on the sequence homology alone, *Aspergillus niger* α-glucosidase is unlikely to pose a risk of food allergenicity.

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[2] http://www.allergenonline.org/index.shtml
[3] http://www.allergenonline.org/databasebrowse.shtml
[4] http://www.allergenonline.org/index.shtml

As for all enzyme products, the Safety Data Sheet (SDS) for the α-glucosidase product would include a precautionary statement that inhalation of enzyme mist/dust may cause allergic respiratory reactions, including asthma, in susceptible individuals on repeated exposure.

6.3.2 Safety of use in food

Alpha-glucosidase from *A. niger* has been determined to be Generally Recognized as Safe (GRAS) by scientific procedures by Danisco US Inc.. Alpha-glucosidase from *A. niger* is described in GRN 246 as being used to form the α-1-6 linkages comprising the isomalto-oligosaccharide mixtures to be used in a variety of foods. The enzymes from *A. niger* have also been evaluated by many other regulatory bodies around the world, and determined to be safe for use in food processing.

In addition to the allergenicity assessment described above, the safety of this α-glucosidase has also been established using the Pariza and Johnson (2001) decision tree:

1. **Is the production strain[5] genetically modified[6, 7]?** Yes. Go to 2.

2. **Is the production strain modified using rDNA techniques?** Yes. Go to 3a.

3a. **Does the expressed enzyme product which is encoded by the introduced DNA[8, 9] have a history of safe use in food[10]?** Yes, *A. niger* transglucosidase activity resulting from the same enzyme protein (GRN 315) is GRAS. Go to 3c.

3c. **Is the test article free of transferable antibiotic resistance gene DNA[11]?**

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[5] Production strain refers to the microbial strain that will be used in enzyme manufacture. It is assumed that the production strain is nonpathogenic, nontoxigenic, and thoroughly characterized; steps 6–11 are intended to ensure this.

[6] The term "genetically modified" refers to any modification of the strain's DNA, including the use of traditional methods (*e.g.*, UV or chemically-induced mutagenesis) or rDNA technologies.

[7] If the answer to this or any other question in the decision tree is unknown, or not determined, the answer is then considered to be NO.

[8] Introduced DNA refers to all DNA sequences introduced into the production organism, including vector and other sequences incorporated during genetic construction, DNA encoding any antibiotic resistance gene, and DNA encoding the desired enzyme product. The vector and othersequences may include selectable marker genes other than antibiotic resistance, noncoding regulatory sequences for the controlled expression of the desired enzyme product, restriction enzyme sites and/or linker sequences, intermediate host sequences, and sequences required for vector maintenance, integration, replication, and/or manipulation. These sequences may be derived wholly from naturally occurring organisms or incorporate specific nucleotide changes introduced by *in vitro* techniques, or they may be entirely synthetic.

[9] If the genetic modification served only to delete host DNA, and if no heterologous DNA remains within the organism, then proceed to step 5.

[10] Engineered enzymes are considered *not* to have a history of safe use in food, unless they are derived from a safe lineage of previously tested engineered enzymes expressed in the same host using the same modification system.

[11] Antibiotic resistance genes are commonly used in the genetic construction of enzyme production strains to identify, select, and stabilize cells carrying introduced DNA. Principles for the safe use of antibiotic resistance genes in the manufacture of food and feed products have been developed (IFBC, 1990; "FDA Guidance for Industry: Use of Antibiotic Resistance Marker Genes in Transgenic Plants

Yes. Transformable DNA was not detected at or above the limit of 5 ng/ml in the enzyme preparation manufactured using this host and production process. Go to 3e.

3e. **Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products?** Yes. Inserted DNA is well characterized Go to 4.

4. **Is the introduced DNA randomly integrated into the chromosome?** Yes. Go to 5.

5. **Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed?** Yes. Although the introduced DNA was randomly integrated (see 4 above), the whole genome of the production strain was sequenced and analyzed to conclude that there is no concern for pleiotropic effects. Go to 6.

6. **Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure[12]?** Yes. The *T. reesei* production strain pertains to the *T. reesei* safe strain lineage (Appendix 8). *T. reesei* safety as a production host and methods of modification are well documented and their safety has been confirmed through toxicology testing.

Conclusion: This test article is accepted.

6.3.3 Safety Studies

All safety studies were conducted in accordance with internationally accepted guidelines (OECD) and compliant with the principles of Good Laboratory Practices ("GLP") per the FDA/OECD.

As mentioned in Section 7.1.2, DuPont Industrial Biosciences (formerly Genencor, a Danisco division) has conducted two safety studies on the *T. reesei* organism itself, one on a recombinant strain derived from RL-P37, modified to overexpress an endoglucanase enzyme, and another on strain A83, also derived from RL-P37 through mutation and selection (see Appendix 8 for summary of the safety studies). We have also conducted many safety studies on *T. reesei* derived enzymes.

A review of all toxicology studies conducted with enzyme preparations produced by different strains of the DuPont Industrial Biosciences *T. reesei* safe strain lineage indicates that, regardless

(http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Biotechnology/ucm096135.htm).

[12] In determining safe strain lineage one should consider the host organism, all of the introduced DNA, and the methods used to genetically modify the host (see text). In some instances, the procedures described by Pariza and Foster (1983) and IFBC (1990) may be considered comparable to this evaluation procedure in establishing a safe strain lineage.

of the production *T. reesei* strain, all enzyme preparations were found to have the following conclusions:

1) Negative as a dermal irritant;
2) Negative as an ocular irritant;
3) Negative as a mutagen, clastogen, and aneugen in genotoxicity studies; and
4) Not observed to adversely affect any specific target organs.

Therefore, due to the consistency of the findings supporting the safety of enzyme preparations derived from different *T. reesei* strains, it is reasonable to expect that most enzyme preparation produced from *T. reesei* strains would have a similar toxicological profile (Appendix 8).

In addition, DuPont Industrial Biosciences has conducted six studies on the α-glucosidase enzyme produced from *T. reesei* TG#626. All the studies were conducted in accordance with the method recommended in the OECD Guideline, OECD Principles of Good Laboratory Practice (GLP) (1997), and all subsequent OECD consensus documents. The results are evaluated, interpreted, and assessed in this document. The test material, Ultra-Filtered Concentrate (UFC), used in all toxicology investigations has the following characteristic:

Lot No.:	08001 UFC
Physical:	Fermentation liquid, brown
Enzyme activity:	5536 MTGU/ml
pH:	5.21
Specific gravity:	1.032 g/ml
Total protein:	106.4 mg/ml
TOS:	12.51 %
	(1 mg Total Protein (TP) = 1.175 mg TOS)

A. *Acute Oral Toxicity Study in Rats, Harlan Labs- Study No. C57481, December 15, 2009*

Procedure:
The objective of this study was to assess the acute toxicity of alpha-glucosidase when administered as a single oral dose followed by a 14-day period of observation. The information is used for both hazard assessment and ranking purposes. The study was initiated with two groups with each group composed of 3 female SPF rats. Alpha-glucosidase was formulated in 0.9% saline solution and administered at a dosing volume of 20 ml/kg (0.1 g total protein/ml). The rats were treated by oral gavage administration at a dosage of 2000 mg total protein/kg bw, which has a corresponding dosage of 2350 mg TOS/kg bw.

Results:
No mortality was observed in this study. There were no overt signs of systemic toxicity throughout the 14-day observation period and at necropsy.

Evaluation:
Under the conditions of this study, the oral LD_{50} was > 2000 mg total protein/kg bw (2350 mg TOS/kg bw). Based on a LD_{50} > 2000 mg /kg bw, alpha-glucosidase is classified as a category 5 (Unclassified - Practically non-toxic) in accordance with the Globally Harmonized System of Classification and Labeling of Chemicals (GHS), 2007.

B. Primary Dermal Irritation study in Rabbits, Harlan Labs-Study No. C5792, November 24, 2009

Procedure:
The objective of this study was to assess the local irritant effects of this α-glucosidase in rabbits (4-hour semi-occlusive application).

The test material was applied by topical semi-occlusive application of 0.5 ml/animal to the intact left flank of each of three young adult New Zealand White rabbits. The duration of treatment was 4 hours. The scoring of skin reactions was performed 1, 24, 48 and 72 hours after removal of the dressing.

Results:
No deaths, overt signs of toxicity, effects on feed consumption, and weight gain were observed. No erythema, eschar or edema was observed at these test sites at any of the examination points throughout the study. The primary irritation score (PIS) for erythema/eschar and edema was 0.0.

Evaluation:
Therefore, on the basis of the results obtained in this study, this alpha-glucosidase is classified as non-irritant per OECD Guideline No 404, "Acute Dermal Irritation/Corrosion", April 2002, and Regulation (EC) No. 1272/2008 of the European Parliament and of the Council of 16 December 2008.

C. Primary Eye Irritation/Corrosion Study in Rabbits, Harlan Labs-Study No. C57514, November 24, 2009

Procedure:
The objective of this study was to assess the ocular irritation potential of this α-glucosidase.

The test material was applied at 0.1 ml to the left eye of each of three young female New Zealand rabbits. Scoring of irritation effects was calculated across three scoring times 24, 48 and 72 hours after instillation. The right eye served as control. After termination of the study, 72 hours after treatment, all animals were sacrificed.

Results:
The individual mean scores for corneal opacity, conjunctivitis, and iritis for all three animals were 0.0.

Evaluation:
The primary eye irritation score was 0.0. Per the Regulation (EC) No. 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labeling and packaging of substances and mixtures, this alpha-glucosidase is classified as "non-irritating to the eyes."

D. Bacterial Reverse Mutation Assay – Ames assay, Harlan Labs, Study No. 1281001, September 28, 2009

Procedure:
The objective of this assay was to assess the potential of this α-glucosidase to induce point mutations (frame-shift and base-pair) in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535, and TA 1537) and *Escherichia coli* strain WP2 uvrA. The test material was tested both in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). A pre-experiment test was performed with strains TA 98, TA 100, TA 1535, TA 1537, and WP2 uvrA. Subsequently, one independent main test was performed with all 5 strains in both the presence and absence of S-9 mix. Triplicate plates were used at each test point. All dose levels were expressed in terms of total protein (TP). Five dose levels of α-glucosidase were used in the main test and ranged from 3 to 5000 µg TP/plate. The highest dose level tested (5000 µg TP/plate) is the maximum required by the OECD guideline. The positive controls used for assays without S-9 mix were sodium azide, 4-nitro-o-phenylene-diamine and methyl methane sulfonate. 2-aminoanthracene was the positive control for assays with S-9 mix. Negative control plates were treated by the addition of sterile deionized water.

Results:
In the screening assay, TrTG was not observed to be toxic to the tested bacteria up to and including the highest dose level (5000 µg TP/plate) in both the absence and presence of S-9 mix. Therefore, 5000 µg TP/plate was selected as the highest dose level for the main test.

In the main test, five dose levels (33, 100, 333, 1000, 2500 and 5000 µg TP/plate) were tested. The plates incubated with the test material showed normal background growth up to 5000 µg/plate with and without metabolic activation. No biologically significant increases in the number of revertant colonies were observed at any dose level of the test item in either main test. There was also no tendency of higher mutation rates with increasing concentrations of the test material. Statistical increases in the number of revertant colonies were noted with the positive controls in both the presence and absence of metabolic activation substantiating the sensitivity of the treatment, plate assay, and efficacy of the metabolic activation mixture.

Evaluation:
Under the conditions of this assay, this α-glucosidase has not shown any evidence of mutagenic activity in the Ames assay. This α-glucosidase did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

E. In vitro Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes, Harlan Labs-Study No. 1281002, November 03, 2009

Procedure:
The objective of this assay was to investigate the potential of this α-glucosidase to induce numerical and/or structural changes in the chromosome of mammalian systems (*i.e.*, human peripheral lymphocytes). In this assay, human lymphocytes were stimulated to divide by the addition of a mitogen (*i.e.,* phytohemagglutinin, PHA). Mitotic activity was noted to start around 40 hours after PHA stimulation and reached a maximum at approximately 3 days.

This α-glucosidase concentrate was mixed with cultures of human peripheral lymphocytes both in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver; S-9 mix). This assay consisted of a preliminary toxicity (dose range finding) assay and one main test. Ten concentrations of this alpha-glucosidase were used in the preliminary assay and at least 3 dose levels were then selected for the two main assays with the highest dose level clearly inducing a toxic effect (50% reduction in mitotic index). Cytotoxicity was characterized by the percentage of mitotic suppression in comparison to the controls by counting 1000 cells per culture in duplicate. In the absence of cytotoxicity, the highest dose selected would be 5000 µg TP/ml, as recommended by the OECD guideline. All dose levels were expressed in terms of total protein (TP).

In the preliminary test, all cultures (with or without S-9 mix) were treated for 4 hours. In the main test, cultures without S-9 mix were treated for 22 hours and those with S-9 mix for 4 hours. Three hours before harvesting, colcemid was added to all cultures at the concentration of 0.2 µg/ml to arrest all cells at the metaphase stage of mitosis. All cultures (with and without S-9 mix) were harvested by centrifugation 22 hours after the start of treatment. The supernatant was discarded, and the cell pellets were re-suspended in a KCl hypotonic solution. The cell suspension was allowed to stand at 37°C for 25 minutes and then centrifuged. The hypotonic solution was then removed. The cells were then fixed on slides, stained, and scored for chromosomal aberrations.

Cytotoxicity was evaluated using the mitotic index (number of cells in mitosis/1000 cells examined). From these results, a dose level causing a decrease in mitotic index of 50% was selected as the highest dose in the main assays. Metaphase analysis (*i.e.*, evaluation of chromosomal aberration) was conducted on at least 100 metaphases per culture dose level. Ethylmethane sulfonate and cyclophosphamide were used as positive controls for cultures without S9 and cultures with S9, respectively.

Results:
Preliminary assay (Experiment I): Ten dose levels were used. Exposure period was 4 hours for both cultures with and without S9 mix. No clear cytotoxicity was observed up to highest concentration tested 5000 µg TP/ml. No visible precipitation of the test material in the culture medium was observed. No biologically relevant increases in cells with chromosomal aberrations were noted in the three highest dose levels selected for analysis. Since the cultures fulfilled the requirements for cytogenicity evaluation, this preliminary assay was designated as Experiment I.

Experiment II: Exposure period was 4 hours for cultures with S9 mix and 22 hours for cultures without S9 mix. No visible precipitation of the test material in the culture medium was observed. In the absence of S9 mix (22-hour cultures); mitotic index was reduced to 53.85 of control after treatment with 5000 µg/ml. In the presence of S9 mix (4-hour cultures), no clear cytotoxicity was observed up to the highest concentration tested, 5000 µg/ml. No biologically relevant increases in cells with chromosomal aberrations were noted in the three highest dose levels selected for analysis. (1632.7, 2857.1 and 5000 µg TP/ml).

In both experiments (I and II) no increase in polyploidy metaphases was observed. In both experiments, significant increases in aberrant metaphases were demonstrated with the positive controls demonstrating the sensitivity of the tests and the efficacy of the S-9 mix.

Evaluation:
Under the conditions of this test, this α-glucosidase did not induce chromosomal aberrations (both structural and numerical) in this *in vitro* cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation up to the highest concentration (5000 µg TP/ml) recommended by guidelines.

F. An 18-week Oral (Gavage) Toxicity Study in Wistar Rats, Harlan Labs- Study No: C57558, April 15, 2010

Procedure:
The objective of this study was to investigate the potential of this α-glucosidase to induce systemic toxicity after repeated daily oral administration to SPF-bred Wistar rats of both sexes. This study lasted 18 weeks and consisted of 2 phases. During the first phase (5-week duration), groups of 10 animals per sex were treated by oral gavage with 0 (0.9% saline), 1, 3 or 6 mg total protein/kg bw/day (corresponding to 0, 1.17, 3.53 or 7.05 mg TOS/kg bw/day, respectively). Due to the absence of overt signs of toxicity in phase 1, both study director and study sponsor agreed to increase the dose levels to 0 (0.9% saline), 10.64, 31.92 and 63.64 mg total protein/kg bw/day (corresponding to 0, 12.5, 31.92 and 63.64 mg TOS/kg bw/day, respectively). The higher dose levels were administered by oral gavage to the rats for an additional 13-week period (phase 2). All animals were sacrificed at termination of phase 2 (week 18 of study).

Animals of the same sex were housed in groups of five in Makrolon-type 4 cages with wire mesh tops and softwood bedding and had access to water (via bottle) and feed *ad libitum*. For environmental enrichment, the animals were provided a supply of Aspen Wood Wool at each change of bedding. All groups were housed under controlled temperature, humidity, and lightning conditions.

All animals were observed daily for mortality and signs of morbidity. Body weight and feed consumption were recorded weekly. Water consumption was recorded twice weekly for each cage. Ophthalmologic examination was performed on all animals prior to study initiation and in the control and high dose groups at study termination. Urinalysis, clinical chemistry, and hematology were conducted after week 5 and 18. A functional observation battery consisting of detailed clinical observation, reactivity to handling and stimuli and motor activity examination was conducted during week 18 for the control and all treated groups. All animals were sacrificed at the end of the 18-week study. After a thorough macroscopic examination, selected organs were removed, weighed, and processed for future histopathologic examination. Microscopic examination was initially conducted on selected organs from control and high dose animals.

Results:
First Phase (5-week duration)
No treatment-related deaths were noted during the 5-week period. There were no treatment-related changes in body weights, feed consumption, and water intake. Hematology and clinical chemistry conducted after 5 weeks of treatment did not reveal any adverse effects.

Second phase (13-week duration):
One control female (# 44) was sacrificed for animal humane reasons on day 113 of the study due to the presence of a mass in the axillary region, which impeded the animal's health. In the low dose group (10.64 mg total protein/kg bw), there was no mortality. In the mid dose group (31.92 mg total protein/kg bw), one male (No. 28) was found dead on day 71 and one female (No. 61) was sacrificed on day 91 for humane reasons. Both deaths were not-treatment related and were likely related to dosing error as indicated by the presence of reddish fluid in the esophagus and lung and perforated esophagus. In the highest dose group (63.64 mg total protein/kg bw), no mortalities were recorded.

There were no biological or statistical differences between the control and treated groups with respect to clinical observation, feed consumption, water consumption, ophthalmologic examinations, body weights, and body weight gains. There were no treatment-related changes in hematology and clinical chemistry at the end of phase 2. There were some alterations in sodium and chloride levels at the highest dose tested (63.64 mg total protein/kg bw) but the changes were still within the historical control data of the laboratory. There were no differences in the functional observation battery, grip strength and locomotor activity assays between treated and control animals.

At necropsy, there were no treatment related findings on organ weights, macroscopic findings, and histopathologic examinations. All microscopic findings were within the background incidence of findings reported in this age and strain of laboratory animals.

Evaluation and conclusion:
Daily administration of this α-glucosidase by oral gavage to Wistar rats at doses of 0, 1, 3, and 6 mg total protein/kg bw (corresponding to 0, 1.17, 3.53 or 7.05 mg TOS/kg bw/day, respectively) for a 5-week period and at doses of 0, 10.64, 31.92 and 63.64 mg total protein/kg bw/day (corresponding to 0, 12.5, 31.92 and 74.8 mg TOS/kg bw/day, respectively) for an additional 13-week period resulted in no treatment-related deaths, clinical observations, feed consumption, body weight changes, hematology, clinical chemistry, urinalysis, organ weights, functional observation, grip strength and locomotor activities. No macroscopic or microscopic changes could be attributed to treatment.

Under the conditions of this assay, the NOAEL (No Observed Adverse Effect Level) was established at the highest dose tested, which was 63.64 mg total protein/kg bw/day, which corresponds to 74.8 mg TOS/kg bw/day.

6.4 OVERALL SAFETY ASSESSMENT

6.4.1 Identification of the NOAEL

In the 90-day oral (gavage) study in Wistar rats, a NOAEL was established at 74.8 mg TOS/kg bw/day. The study was designed based on OECD guideline No. 408 and conducted in compliance with both the FDA Good Laboratory Practice Regulations and the OECD Good Laboratory Practice. Since human exposure to this α-glucosidase is through oral ingestion, selection of this NOAEL is thus appropriate.

NOAEL = 74.8 mg TOS/kg bw/day

6.4.2 Conclusion

Determination of the margin of safety

The margin of safety was calculated by dividing the NOAEL obtained from the 90-day oral (gavage) study in rats by the human exposure (worst case scenario) assessed in section 3. This worst-case assessment takes into account the exposure from consumption of food processed with both the alpha-glucosidase enzyme activity and transglucosidase previously notified as GRAS to FDA (GRN 315).

$$\text{Margin of safety} = \frac{\text{No observed adverse effect level}}{\text{Maximum daily exposure}}$$

$$\text{Margin of safety} = \frac{74.8 \text{ mg TOS/kg bw/day}}{0.443 \text{ mg TOS/kg bw/day}} = 169$$

6.5 BASIS FOR GENERAL RECOGNITION OF SAFETY

As noted in the Safety sections above, *Trichoderma reesei*, and enzyme preparations derived there from, including glucoamylase, cellulase, beta-glucanase, xylanase, acid fungal protease, chymosin and α-amylase enzyme preparations, are well recognized by qualified experts as being safe for their intended uses. Published literature, government laws and regulations, reviews by expert panels such as FAO/WHO JECFA (1992), as well as Dansico US Inc.'s (operating as DuPont Industrial Biosciences) own unpublished safety studies, support such a conclusion.

Trichoderma reesei is widely used by enzyme manufacturers around the world for production of enzyme preparations for use in human food, animal feed, and numerous industrial enzyme applications. It is generally recognized as a safe host for enzyme production. In addition, the *T. reesei* lineage used by Genencor has been demonstrated to be safe.

The safety of *A. niger* α-glucosidase expressed in *T. reesei* strain as a processing aid to be used in the manufacture of the following products at the maximum recommended application rates supported by toxicological data:

1) Potable alcohol from starch and the production of organic acids (*e.g.,* lactic acid, citric acid and succinic) from α-glucosidase activity;
2) monosodium glutamate (MSG) from α-glucosidase activity;
3) production of IMO syrups from starch and potable ethanol from molasses from transglucosidase activity (FDA GRN 315).

The margin of safety was calculated to be 169 based on a NOAEL of 74.8 mg TOS/kg bw/day (obtained from the cumulative maximum daily exposure to both transglucosidase and α-glucosidase of 0.443 mg TOS/kg bw/day). In the rare case of ingestion of the transglucosidase/α-glucosidase enzyme preparation, it poses no safety or health concerns to humans, based on maximum recommended application rates which are supported by existing toxicology data for this enzyme. Based on a margin of safety greater than 100 even in the worst-case, the uses of α-glucosidase as a processing aid in the manufacture of potable alcohol from starch, organic acids, and monosodium glutamate are not of human health concern.

Based on the publicly available scientific data from the literature and additional supporting data generated by Danisco US Inc. (operating as DuPont Industrial Biosciences), the company has concluded that alpha-glucosidase from *Trichoderma reesei* strain TG #626 is safe and suitable for use in the manufacture of potable alcohol from starch, organic acids (*e.g.,* lactic acid, citric acid, and succinic acid), and monosodium glutamate. Therefore, it can be considered Generally Recognized as Safe (GRAS). In addition, the safety determination, including construction of the production organism, the production process and materials, and safety of the product, were reviewed by an external expert in the field, Dr. Michael Pariza, who concurred with the company's conclusion that the product is GRAS (see Appendix 9).

7. SUPPORTING DATA AND INFORMATION

7.1 LIST OF REFERENCES

Aunstrup, K., Andersen, O., Falch, E. A., and Nielsen, T. K. 1979. Production of Microbial Enzymes in Microbial Technology, 2nd ed., Volume 1. Eds. Peppler, H.J., and Perlman, D., Chapter 9, pp. 282-309.

Aunstrup, K. 1979. Production, Isolation, and Economics of Extracellular Enzymes in Applied Biochemistry and Bioengineering, Volume 2, Enzyme Technology, Eds. Wingard, L.B., Katchalski-Katzir, E. and Golsdstein, L. pp. 28-68.

Berkowitz, D. and Maryanski, J. 1989. Implications of biotechnology on international food standards and codes of practice. Joint FAO/WHO Food Standards Program, Codex Alimentarius Commission, Eighteenth Session, Geneva, July 3-12.

Bindslev-Jensen C., P.S. Skov, E.L. Roggen, P. Hvass, D.S. Brinch. 2006. Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry. *Food Chem. Toxicol*. 44: 1909-1915.

Bissett, J. 1984. A revision of the genus *Trichoderma*. I. Section *Longibrachiatum* sect. Nov. Can. J. Bot. 62: 924-931.

Blumenthal. C. 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. *Regul. Toxicol. Pharmacol*. 39: 214-228.

Brückner, H. and Graf, H. 1983. Paracelsin, a peptide antibiotic containing alpha-aminoisobutyric acid, isolated from *Trichoderma reesei* Simmons. Part A. Experientia 39:528-530.

Committee on Food Chemicals Codex. 2009. Food Chemicals Codex. 6th Ed. National Academic Press: Washington, D.C.

DUAN, K. J., SHEU, D. C., & LIN, C. T. 1995. Transglucosylation of a Fungal α-Glucosidase. Annals of the New York Academy of Sciences, *750*(1), 325-328.

Dugan, F.M. 1998. A Note on *Trichoderma reesei* and *T. longibrachiatum*, United States Federation for Culture Collections Newsletter. 28(2): 1- 2.

Enzyme Applications in Encyclopedia of Chemical Technology, 4th edition. 1994. Kroschwitz, J.I., Volume 9, pp. 567-620.

Environmental Protection Agency (EPA). 1997. TSCA section 5(H) (4) exemption for *Aspergillus niger*. Item #3170.

Environmental Protection Agency (EPA). 2012. Microorganisms; General Exemptions From Reporting Requirements; Revisions to Recipient Organisms Eligible for Tier 1 and Tier II Exemption. Federal Register / Vol. 77, No. 172. 54499-54411

EU Scientific Committee for Food (SCF). 1991. Guidelines for the presentation of data on food enzymes. Reports of the Scientific Committee for Food, 27th series.

Evaluation of certain food additives and naturally occurring toxicants (Thirty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). 1992. WHO Technical Report Series No. 828.

FAO/WHO. 1996. Biotechnology and Food Safety, Report of a Joint FAO/WHO Consultation. FAO Food and Nutrition Paper 61. Rome, Italy.

Food and Drug Administration (FDA). 1996. Carbohydrase and cellulase derived from *Aspergillus niger*. 21 C.F.R. Parts 173.120. National Archives and Records Administration: Washington DC.

Food and Drug Administration (FDA). 1996. Solvent extraction process for citric acid. 21 C.F.R. Parts 173.280. National Archives and Records Administration: Washington DC.

GRAS Notice Inventory,
http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=grasListing

Herman, RA, Ping Song, and A ThirumalaiswamySekhar. 2009. Value of eight-amino-acid matches in predicting the allergenicity status of proteins: an empirical bioinformatic investigation. *Clinical and Molecular Allergy* 7: 9

IFBC (International Food Biotechnology Council). 1990. Chapter 4: Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms in Biotechnologies and Food: Assuring the Safety of Foods Produced by Genetic Modification. *Regul. Toxicol. Pharmacol.* 12: S1-S196.

IUBMB. Enzyme nomenclature. Recommendations 1992.
http://www.chem.qmw.ac.uk/iubmb/enzyme; 2001.

Joint FAO/WHO Expert Committee on Food Additives (JECFA). 1990. Specifications for identity and purity of certain food additives. FAO food additives series #26. FAO.

Khuls, K, Lieckfeldt, E., Samuels, G.J., Kovacs, W., Meyer, W., Petrini, O., Gams, W., Börner, T. and Kubicek, C.P. 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.

Kimura, A, Takata, M, Sakai, O, Matsui, H, Takai, N, Takayanagi, T, Nishimura, I, Uozumi, T, Chiba, S. 1992. Complete amino acid sequence of crystalline alpha-glucosidase from *Aspergillus niger*., *Biosci. Biotechnol. Biochem*. 56: 1368-1370.

Kroschwitz, J.I. 1994 Enzyme Applications in Encyclopedia of Chemical Technology, 4th edition. Volume 9, pp. 567-620.

Ladics, G. S., Cressman, R. F., Herouet-Guicheney, C., Herman, R. A., Privalle, L., Song, P., & McClain, S. 2011. Bioinformatics and the allergy assessment of agricultural biotechnology products: industry practices and recommendations. Regulatory Toxicology and Pharmacology, 60(1), 46-53.

Meyer, W., Morawetz, R., Borner, T. and Kubicek, C. P. 1992. The use of DNA-fingerprint analysis in the classification of some species of the *Trichoderma* aggregate. *Curr. Genet*. 21: 27-30.

Nakamura, A., Nishimura, I., Yokoyama, A., Lee, D.G., Hidaka, M., Masaki, H., Kimura, A., Chiba, S. and Uozumi, T. 1997. Cloning and sequencing of an alpha-glucosidase gene from *Aspergillus niger* and its expression in *A. nidulans*, *J. Biotechnol.* 53: 75-84.

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

Olempska-Beer, Z.S., Merker, R.I., Ditto, M.D., and DiNovi, M.J. ,2006. Food-processing enzymes from recombinant microorganisms—a review. *Regul. Toxicol. Pharmacol.* 45: 144-158.

Organization for Economic Cooperation and Development (OECD). 1993. Safety Evaluation of Foods Derived by Modern Biotechnology.

Pariza, M. W. and Johnson, E. A. 2001. Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing – Update for a New Century. *Regul. Toxicol. Pharmacol.* 33: 173-186.

Pariza, M.W. and Foster, E. M. 1983. Determining the Safety of Enzymes Used in Food Processing. *J. Food Prot.,* 46: 453-468.

Samuels, G.J., Ismaiel, A., Mulaw, T.B., Szakacs, G., Druzhinina, I.S., Kubicek, C.P. and Jaklitsch, W.M., 2012. The Longibrachiatum Clade of Trichoderma: a revision with new species. Fungal Diversity, 55(1), pp.77-108.

Sewalt, V, Shanahan, D, Gregg, L, La Marta, J, and Carrillo, R. 2016. The Generally Recognized as Safe (GRAS) Process for Industrial Microbial Enzymes. *Industrial Biotechnology*. 12(5): 295-302.

Schuster, E., Dunn-Coleman, N., Frisvad, J.C., van Dijck, P.W.M. 2002. On the safety of *Aspergillus niger*—a review. *Appl. Microbiol. Biotechnol.* 59: 426–435.

Sheir-Neiss, G. and Montenecourt, B.S. 1984. Characterization of the secreted cellulases of *Trichoderma reesei* wild type and mutants during controlled fermentations. *Appl. Microbiol. Biotechnol.* 20: 46-53.

Solfrizzo, M., Altomare, C., Visconti, A., Bottalico, A. and Perrone, G. 1994. Detection of peptaibols and their hydrolysis products in cultures of *Trichoderma* species. *Nat. Toxins* 2: 360-365.

Uhlig, H. Industrial enzymes and their applications. 1998. Translated by Linsmaier-Bednar, E. M. John Wiley & Sons, Inc.: New York.

U.S. Pharmacopeia, 2016. Food Chemicals Codex (FCC), 10th Edition. United States Pharmacopeial Convention (USP), Rockville, MD.

Yoshikawa, K, Yamamoto, K, Okada, S. 1994. Classification of some alpha-glucosidases and alpha-xylosidases on the basis of substrate specificity, *Biosci. Biotechnol. Biochem.* 58: 1392-1398.

7.2 LIST OF APPENDICES

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Appendix 1: FDA GRN 315 "No Question" Letter

DEPARTMENT OF HEALTH & HUMAN SERVICES Public Health Service

Food and Drug Administration
College Park, MD 20740

Vincent Sewalt, Ph. D.
Director of Regulatory Affairs
Genencor, a Danisco Division
925 Page Mill Road
Palo Alto, CA 94304

Re: GRAS Notice No. GRN 000315

Dear Dr. Sewalt:

The Food and Drug Administration (FDA) is responding to the notice, dated December 04, 2009, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on December 7, 2009, filed it on January 6, 2010, and designated it as GRAS Notice No. GRN 000315.

The subject of the notice is a transglucosidase enzyme preparation from *Trichoderma reesei* expressing the gene encoding transglucosidase from *Aspergillus niger*. The notice informs FDA of the view of Genencor, a Danisco Division (Genencor) that the transglucosidase enzyme preparation is GRAS, through scientific procedures, for use in the production of isomalto-oligosaccharide syrups from starch and potable ethanol from molasses.

Commercial enzyme preparations that are used in food typically contain an enzyme component, which catalyzes the chemical reaction that is responsible for its technical effect, as well as substances used as stabilizers, preservatives or diluents. Enzyme preparations may also contain constituents derived from the production organism and manufacturing process. In its notice, Genencor provides information about all the components of the transglucosidase enzyme preparation.

Genencor's transglucosidase catalyzes both the hydrolysis and transfer of D-glucosyl units of oligosaccharides and converts 1,4 glucosidic linkages to 1,6 glucosidic linkages. When used in the production of isomalto-oligosaccharide syrups from starch, transglucosidase converts malto-oligosaccharides to isomalto-oligosaccharides. In molasses, transglucosidase converts non-fermentable sugars such as raffinose and stachyose to sucrose, galactose, glucose and fructose, which can then be fermented into alcohol.

According to the classification system of enzymes established by the International Union of Biochemistry and Molecular Biology (IUBMB), transglucosidase is identified by the Enzyme Commission number 2.4.1.24. Its accepted name is 1,4-α-glucan 6-α- glucosyltransferase, and its systematic name is 1,4-α-D-glucan:1,4-α-D-glucan (D-glucose)-6-α-D-glucosyltransferase. The Chemical Abstract Service Registry number for transglucosidase is 9030-12-0.

Genencor states that the production organism, *T. reesei*, is non-toxigenic and non-pathogenic and has a long history of safe use in industrial scale enzyme production. In the literature search conducted by Genencor, no reports were found that implicate *T. reesei* in disease, toxicity, or allergenicity among healthy adults. *T. reesei* is classified as a Biosafety Level 1 (BSL1) microorganism by the American Type Culture Collection (ATCC). BSL1 microorganisms are not known to cause disease in healthy humans.

According to Genencor, the production strain *T. reesei* TG #626, GICC 03289 was derived by recombinant DNA methods from *T. reesei* strain RL-P37. *T. reesei* strain RL-P37 is a commercial production strain that was derived from the well-known wild-type *T. reesei* strain QM6a using several classical mutagenesis steps. *T. reesei* strain RL-P37 has been used by Genencor for production of cellulases for over fifteen years and has been determined to be non-pathogenic and non-toxicogenic via intraperitoneal dosing in rats. Genencor also notes that FDA issued a "no questions" letter in response to GRAS Notice No. 000230 containing information on bovine chymosin obtained from a *T. reesei* production strain, which was derived from *T. reesei* strain RL-P37.

Genencor states that the transglucosidase production strain was constructed by transformation of *T. reesei* strain RL-P37 with the expression vector containing DNA encoding the mature transglucosidase from *A. niger* and the *amdS* selectable marker gene from *Aspergillus nidulans*. The vector was integrated into the chromosomal DNA of the recipient strain and the final construct was verified by Southern blot analysis. The production strain was shown to be stable for at least 60 generations of fermentation, based on transglucosidase production. Genencor states that no antibiotic resistance genes were used in the construction of the production microorganism. Genencor also verified the absence of the production microorganism in the final enzyme product.

Genencor states that the donor organism, *A. niger*, has a long history of safe use as a source of enzymes and is currently used to produce enzymes, such as protease and glucoamylase. Genencor cites previous use of *A. niger* transglucosidase in the production of isomalto-oligosaccharides (GRN 000246) as further evidence of its safety.

According to Genencor, transglucosidase is manufactured by submerged straight-batch or fed-batch pure culture fermentation of the *T. reesei* production strain. The production process is consistent with FDA's current food good manufacturing practice (cGMP) as set forth in 21 CFR Part 110. Each production batch is initiated from a stock culture, which is tested prior to use for identity, absence of contaminating microorganisms and enzyme generating ability. The fermentation process is conducted under controlled conditions and the fermentation broth is periodically tested for microbial contamination. Transglucosidase is secreted to the fermentation broth and is subsequently recovered via several purification and concentration steps. The resulting liquid concentrate is formulated with sodium chloride and glycerol. The finished enzyme preparation contains approximately 3 percent transglucosidase, 3 percent sodium chloride, 50 percent glycerol and 44 percent water. Genencor states that this product meets the purity specifications for enzyme preparations set forth in the Food Chemicals Codex (6th Edition) and the General Specifications and Considerations for Enzyme Preparations Used in Food Processing established by the FAO/WHO Joint Expert Committee on Food Additives.

Genencor states that the transglucosidase enzyme preparation will be used in the production of isomalto-oligosaccharides at use levels from 0.5 to 1.5 kg per ton of starch dry solids. After two days of reaction, the enzyme is inactivated and removed from the final product via several purification steps. In the production of potable ethanol, the transglucosidase enzyme preparation

will be added during fermentation at a level of 6-20 mg/kg. Genencor states that the enzyme will be inactivated and removed from the final potable ethanol product during distillation of the fermentation broth.

To estimate human exposure to the transglucosidase enzyme preparation, Genencor assumed that the enzyme preparation would be used at the highest use level and all would be carried over to the final food product. In such a case, the cumulative exposure to transglucosidase from consumption of both potable ethanol and isomalto-oligosaccharides would be 0.034 mg enzyme per kg body weight per day (mg/kg bw/d) expressed as total protein or 0.040 mg/kg bw/day expressed as total organic solids (TOS), assuming a body weight of 60 kg.

Genencor provided the results of toxicological studies for eight enzyme preparations derived from *T. reesei*, one from a traditionally modified strain and seven from recombinant strains. Toxicology tests included 91-day subchronic feeding studies in rats for seven enzyme preparations and a 28-day study for one enzyme preparation. The studies also included a bacterial reverse mutation assay and an *in vitro* chromosomal aberration assay with either human lymphocytes or Chinese hamster ovary cells. All enzyme preparations were found to be non-toxic, non-mutagenic, and not clastogenic. Genencor concludes that the results of these studies as well as pathogenicity studies conducted on *T. reesei* confirm that *T. reesei* strain RL-P37 is a safe production host and the enzyme preparations resulting from it are safe for use in food.

Genencor cites published information in support of the notion that the ingestion of food enzymes in general is not considered to be a concern with regard to food allergy. Genencor reports that consistent with the recommendations of the Codex Alimentarius Commission (2003), Genencor conducted the amino acid sequence homology search for transglucosidase from *A. niger* with known allergens listed in two publicly available allergen databases. Genencor identified no homology of 35% or greater when using an 80-amino acid sliding window and no homology with 8-amino acid stretches. Genencor found one 7-amino acid match and five 6-amino acid matches; however, after analyzing these matches, Genencor eliminated them as potential allergen binding epitopes. Genencor concludes that transglucosidase is unlikely to pose a risk of food allergy.

Section 301(ll) of the Federal Food, Drug, and Cosmetic Act (FFDCA)

Section 301(ll) of the FFDCA prohibits the introduction or delivery for introduction into interstate commerce of any food that contains a drug approved under section 505 of the FFDCA, a biological product licensed under section 351 of the Public Health Service Act, or a drug or a biological product for which substantial clinical investigations have been instituted and their existence made public, unless one of the exemptions in section 301(ll)(1)-(4) applies. In its review of Genencor's notice that the transglucosidase enzyme preparation is GRAS for use in the production of isomalto-oligosaccharide syrups and potable ethanol, FDA did not consider whether section 301(ll) or any of its exemptions apply to foods containing the transglucosidase enzyme preparation. Accordingly, this response should not be construed to be a statement that foods that contain the transglucosidase enzyme preparation, if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusions

Based on the information provided by Genencor, as well as other information available to FDA, the agency has no questions at this time regarding Genencor's conclusion that the transglucosidase enzyme preparation is GRAS under the intended conditions of use. The agency has not, however,

made its own determination regarding the GRAS status of the subject use of the transglucosidase enzyme preparation. As always, it is the continuing responsibility of Genencor to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter responding to GRN 000315, as well as a copy of the information in this notice that conforms to the information in the GRAS exemption claim (proposed 21 CFR 170.36(c)(1)), is available for public review and copying via the FDA home page at http://www.fda.gov. To view or obtain an electronic copy of the text of the letter, follow the hyperlinks from the "Food" topic to the "Food Ingredients and Packaging" section to the "Generally Recognized as Safe (GRAS)" page where the GRAS Inventory is listed.

Sincerely,

(b) (6)

Mitchell A. Cheeseman, Ph.D.
Acting Director
Office of Food Additive Safety
Center for Food Safety
and Applied Nutrition

Appendix 2: FDA Response Letter for the Identity Name of the Enzyme

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
College Park, MD 20740

June 17, 2011

Vincent Sewalt, Ph. D.
Director of Regulatory Affairs
Genencor, a Danisco Division
925 Page Mill Road
Palo Alto, CA 94304

Re: GRAS Notice No. GRN 000315

Dear Dr. Sewalt:

The Food and Drug Administration (FDA) is responding to the supplement dated March 28, 2011, that you submitted on behalf of Genencor, a Danisco Division (Genencor), regarding the renaming of the enzyme described in GRAS Notice No. GRN 000315. Genencor initially submitted GRAS Notice No. GRN 000315 in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS) (the GRAS proposal). FDA received this notice on December 9, 2009, filed it on January 6, 2010, and designated it as GRAS Notice No. GRN 000315.

The subject of the notice is transglucosidase enzyme preparation from *Trichoderma reesei* expressing the gene encoding transglucosidase from *Aspergillus niger* (transglucosidase enzyme preparation). The notice informed FDA of the view of Genencor that the transglucosidase enzyme preparation is GRAS, through scientific procedures, for use in the production of isomalto-oligosaccharide syrups from starch and potable ethanol from molasses. In a letter dated May 5, 2010, FDA informed Genencor that the agency had no questions at that time regarding Genencor's conclusion that the subject of the notice is GRAS under the intended conditions of use.

In the supplement dated March 28, 2011, Genencor informed FDA that transglucosidase (EC 2.4.1.24) from *A. niger* also has α-glucosidase activity (EC 3.2.1.20). Genencor states that the correct name of the enzyme that is the subject of GRN 000315 should be α-glucosidase, even though the enzyme also displays transglucosidase activity. Genencor based this on the sequence identity of transglucosidase with that of α-glucosidase identified in GenBank (GenBank: AA23616).

FDA reviewed the supplement and acknowledges the dual activity of this enzyme. While the agency will include this supplement in the record, it will continue to refer to the subject of GRN 000315 as transglucosidase enzyme preparation. FDA believes that this is appropriate given the intended use of transglucosidase enzyme preparation is based on transglucosidase activity. Consequently, the agency will refer to the subject of GRN 000315 as "transglucosidase enzyme preparation."

As always, it is the continuing responsibility of Genencor to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter responding to the supplement to GRN 000315, as well as a copy of the information in this notice that conforms to the information in the GRAS exemption claim (proposed 21 CFR 170.36(c)(1)), is available for public review and copying at www.fda.gov/grasnoticeinventory

Sincerely

(b) (6)

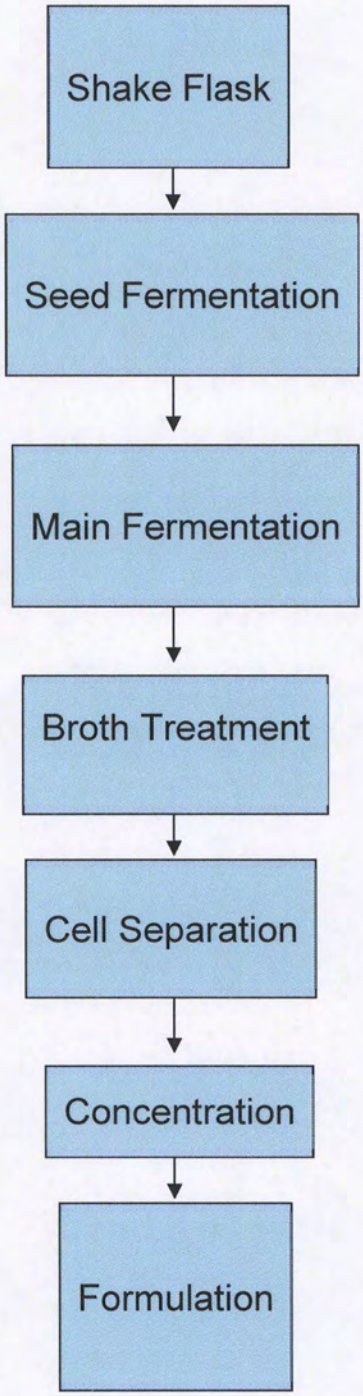
Antonia Mattia, Ph.D.
Director
Division of Biotechnology
and GRAS Notice Review
Office of Food Additive Safety
Center for Food Safety
and Applied Nutrition

Appendix 3: ETA Published Paper on GRAS for Enzyme by Sewalt *et. al.*

Appendix 4: Amino Acid Sequence of Alpha-glucosidase

STTAPSQPQFTIPASADVGAQLIANIDDPQAADAQSVCPGYKASKVQHNSRGFTASLQLAGRPCNVYGTDVESLTLS
VEYQDSDRLNIQILPTHVDSTNASWYFLSENLVPRPKASLNASVSQSDLFVSWSNEPSFNFKVIRKATGDALFSTEG
TVLVYENQFIEFVTALPEEYNLYGLGEHITQFRLQRNANLTIYPSDDGTPIDQNLYGQHPFYLDTRYYKGDRQNGSY
IPVKSSEADASQDYISLSHGVFLRNSHGLEILLRSQKLIWRTLGGGIDLTFYSGPAPADVTRQYLTSTVGLPAMQQY
NTLGFHQCRWGYNNWSDLADVVANFEKFEIPLEYIWTDIDYMHGYRNFDNDQHRFSYSEGDEFLSKLHESGRYYVPI
VDAALYIPNPENASDAYATYDRGAADDVFLKNPDGSLYIGAVWPGYTVFPDWHHPKAVDFWANELVIWSKKVAFDGV
WYDMSEVSSFCVGSCGTGNLTLNPAHPSFLLPGEPGDIIYDYPEAFNITNATEAASASAGASSQAAATATTTSTSVS
YLRTTPTPGVRNVEHPPYVINHDQEGHDLSVHAVSPNATHVDGVEEYDVHGLYGHQGLNATYQGLLEVWSHKRRPFI
IGRSTFAGSGKWAGHWGGDNYSKWWSMYYSISQALSFSLFGIPMFGADTCGFNGNSDEELCNRWMQLSAFFPFYRNH
NELSTIPQEPYRWASVIEATKSAMRIRYAILPYFYTLFDLAHTTGSTVMRALSWEFPNDPTLAAVETQFMVGPAIMV
VPVLEPLVNTVKGVFPGVGHGEVWYDWYTQAAVDAKPGVNTTISAPLGHIPVYVRGGNILPMQEPALTTREARQTPW
ALLAALGSNGTASGQLYLDDGESIYPNATLHVDFTASRSSLRSSAQGRWKERNPLANVTVLGVNKEPSAVTLNGQAV
FPGSVTYNSTSQVLFVGGLQNLTKGGAWAENWVLEW

Appendix 5: Manufacturing Process of Alpha-glucosidase



Appendix 6: Certificate of Analysis (3 lots)

CERTIFICATE OF ANALYSIS

PRODUCT: **FERMENZYME T**

BATCH: **1682630349**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITIES			
Alpha-glucosidase	U/g	1650-2140	1800
PHYSICAL PROPERTIES			
pH		4.0-5.0	4.2
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/ml	0-50000	<1000
Total Coliforms	CFU/ml	0-30	<10
E. coli	/25ml	Negative by test	Negative
Salmonella	/25ml	Negative by test	Negative
Production Strain	/ml	Negative by test	Negative
Antibacterial Activity	/ml	Negative by test	Negative
OTHER ASSAYS			
Arsenic	mg/kg	0-3	<3
Cadmium	mg/kg	0-0.5	<0.5
Mercury	mg/kg	0-0.5	<0.5
Lead	mg/kg	0-5	<5
Mycotoxins		Negative by test	Negative

27-Jan-2017
Date

Kelly A. Altman
Manager, Quality Assurance

This certificate of analysis was electronically generated and therefore has not been signed.

CERTIFICATE OF ANALYSIS

PRODUCT: **FERMENZYME T**

BATCH: **1682681588**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITIES			
Alpha-glucosidase	U/g	1650-2140	1800
PHYSICAL PROPERTIES			
pH		4.0-5.0	4.2
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/ml	0-50000	<1000
Total Coliforms	CFU/ml	0-30	<10
E. coli	/25ml	Negative by test	Negative
Salmonella	/25ml	Negative by test	Negative
Production Strain	/ml	Negative by test	Negative
Antibacterial Activity	/ml	Negative by test	Negative
OTHER ASSAYS			
Arsenic	mg/kg	0-3	<3
Cadmium	mg/kg	0-0.5	<0.5
Mercury	mg/kg	0-0.5	<0.5
Lead	mg/kg	0-5	<5
Mycotoxins		Negative by test	Negative

27-Jan-2017
Date

Kelly A. Altman
Manager, Quality Assurance

This certificate of analysis was electronically generated and therefore has not been signed.

CERTIFICATE OF ANALYSIS

PRODUCT: **FERMENZYME T**

BATCH: **1682727203**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITIES			
Alpha-glucosidase	U/g	1650-2140	1800
PHYSICAL PROPERTIES			
pH		4.0-5.0	4.3
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/ml	0-50000	<1000
Total Coliforms	CFU/ml	0-30	<10
E. coli	/25ml	Negative by test	Negative
Salmonella	/25ml	Negative by test	Negative
Production Strain	/ml	Negative by test	Negative
Antibacterial Activity	/ml	Negative by test	Negative
OTHER ASSAYS			
Arsenic	mg/kg	0-3	<3
Cadmium	mg/kg	0-0.5	<0.5
Mercury	mg/kg	0-0.5	<0.5
Lead	mg/kg	0-5	<5
Mycotoxins		Negative by test	Negative

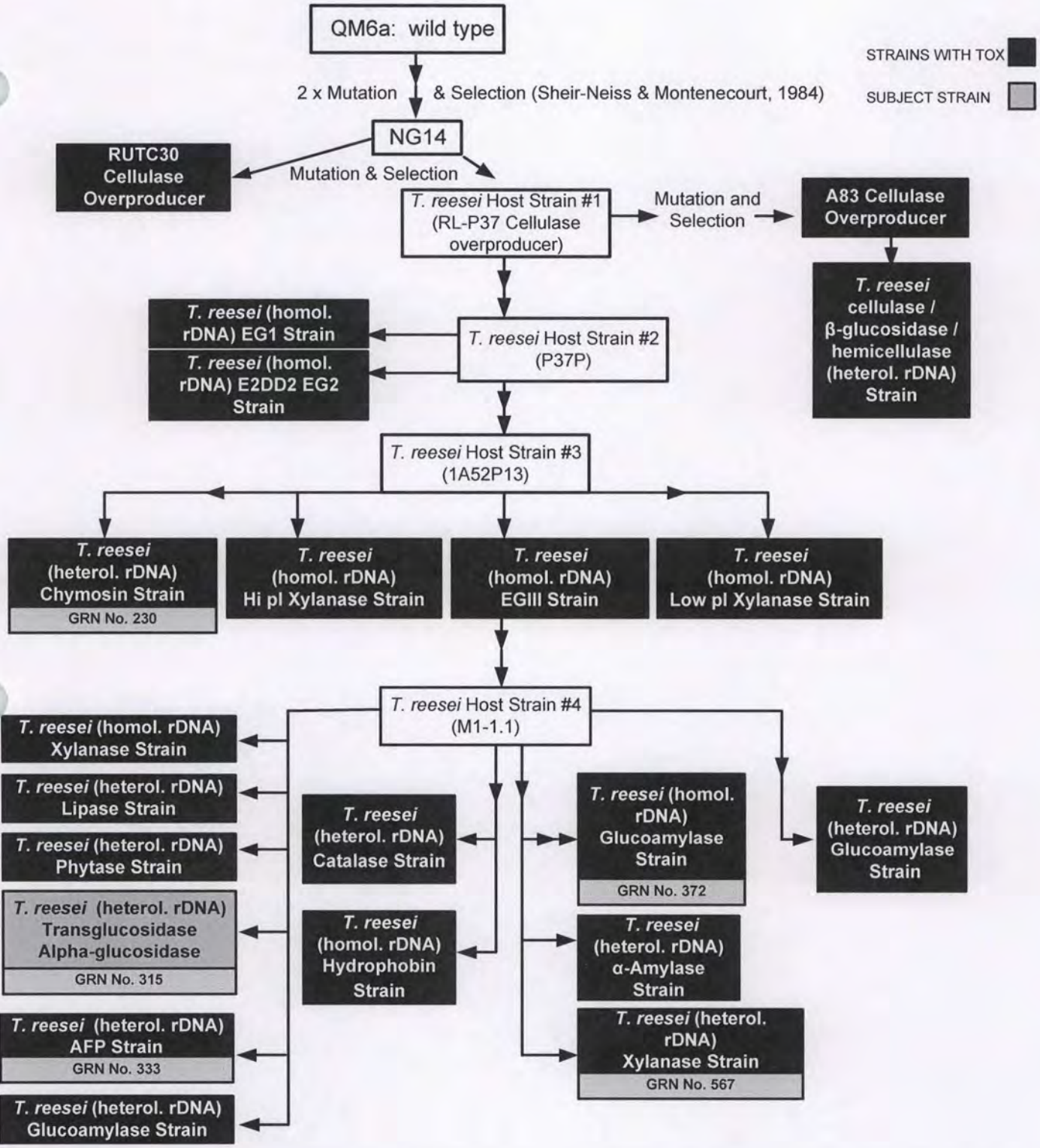
27-Jan-2017
Date

Kelly A. Altman
Manager, Quality Assurance

This certificate of analysis was electronically generated and therefore has not been signed.

Appendix 7: *T. reesei* taxonomy articles

Appendix 8: *T. reesei* Strain Lineage and Summary of Safety Studies



Most enzymes derived from this Safe Strain Lineage were determined to be GRAS for their intended use, with GRAS Notices reviewed by the US FDA for enzymes from strains designated with horizontal banners indicating the GRAS Notice number.

The subject strain of this submission is the alpha-glucosidase/transglucosidase producing strain indicated by the gray color.

The safety of the alpha-glucosidase/transglucosidase enzyme is fully supported by repeated testing of other enzymes produced by members of this Safe Strain Lineage. The black colored boxes indicate strains for which we conducted toxicology tests.

The NOAEL for the alpha-glucosidase/transglucosidase is used to calculate its safety margin in the intended use

Summary of safety studies on *Trichoderma reesei* derived enzymes in support of DuPont/Genencor's Safe Strain Lineage

Toxicology Test Summaries

The safety of the 20 enzyme preparations derived from the 20 recombinant production strains were assessed in a number of toxicology tests as shown in the table below. The table also includes the toxicology tests for two non-recombinant *T. reesei* strains (RUT C30 and A83) and/or product derived from them. All enzyme preparations were found to be non-toxic, non-mutagenic and not clastogenic.

PRODUCTION ORGANISM	ENZYME	TOXICOLOGY TEST	RESULT
I. *T. reesei* A83 (Traditionally modified)	Cellulase	Pathogenicity study, rats	Non-pathogenic Non-toxicogenic
		91-day subchronic oral toxicity study, rats	No adverse effect
		Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosomal aberration assay, human lymphocytes	Not clastogenic
II. *T. reesei* RUT C30 (Traditionally modified)	Cellulase	90-day feeding study, rats	No adverse effects
		Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosomal aberration assay, human lymphocytes	Not clastogenic
III. *T. reesei* (heterologous rDNA)	Endoglucanase I	14-day oral feeding study, rats	No adverse effects
		Pathogenicity study, rats	Non pathogenic
		91-day subchronic oral toxicity study, rats	No adverse effects
		In vitro chromosome assay, human lymphocytes	Not clastogenic
IV. *T. reesei* (heterologous rDNA)	High pI Xylanase	91-day subchronic oral toxicity study, rats	No adverse effects
		Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosomal aberration assay with	Not clastogenic

		Chinese Hamster Ovary (CHO) cells	
V. *T. reesei* (heterologous rDNA)	Endoglucanase II	90-day repeated dose oral (gavage) toxicity study in the rat	No adverse effects
		In vitro chromosomal aberration assay, human lymphocytes	Not clastogenic
		Bacterial reverse mutation assay (Ames)	Not mutagenic
VI. *T. reesei* (heterologous rDNA)	Endoglucanase III	28-Day subacute oral toxicity study, rats	No adverse effects
		Bacterial reverse mutation assay (Ames)	Not mutagenic
VII. *T. reesei* (heterologous rDNA)	Low pI Xylanase	91-day subchronic oral toxicity study, rats	No adverse effects
		Bacterial reverse mutation assay (Ames)	Not mutagenic
		In vitro chromosomal aberration assay, human lymphocytes	Not clastogenic
VIII. *T. reesei* (heterologous rDNA)	Xylanase	91-day subchronic oral toxicity study, rats	No adverse effects
		Bacterial reverse mutation assay (Ames)	Not mutagenic
		In vitro chromosomal aberration assay, human lymphocytes	Not clastogenic
IX. *T. reesei* (heterologous rDNA)	Protease	13-week oral (gavage) toxicology studies, rats	No adverse effects
		Bacterial reverse mutation assay (Ames)	Not mutagenic
		In vitro chromosomal aberration assay, human lymphocytes	Not clastogenic
X. *T. reesei* (heterologous rDNA)	Phosphatase (Phytase)	A 13-week Oral (Gavage) Toxicity Study in Rats	No adverse effects
		Bacterial reverse mutation assay (Ames)	Not mutagenic
		In vitro Mammalian Chromosomal Aberration Test	Not clastogenic

		Performed with Human Lymphocytes	
XI. *T. reesei* (heterologous rDNA)	Chymosin	Bacterial reverse mutation assay (Ames)	Not mutagenic
		I In vitro Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes.	Not clastogenic
		A 13-week Oral (Gavage) Toxicity Study in Rats	No adverse effects detected
XII. *T. reesei* (heterologous rDNA)	Alpha-Glucosidase/ Transglucosidase	Bacterial reverse mutation assay (Ames)	Not mutagenic
		In vitro Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes.	Not clastogenic
		18-week Oral (Gavage) Toxicity Study in Wistar Rats	No adverse effects
XIII. *T. reesei* (heterologous rDNA)	Glucoamylase	Bacterial reverse mutation assay (Ames)	Not mutagenic
		In vitro Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes	Not clastogenic
		90-day oral (gavage) toxicology study, rats	No adverse effects
XIV. *T. reesei* (heterologous rDNA)	Lipase	Bacterial reverse mutation assay (Ames)	Not mutagenic
		In vitro Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes	Not clastogenic
		13-week Oral (Gavage) Toxicity Study in Wistar Rats	No adverse effects
XV. *T. reesei* (heterologous rDNA)	Alpha-amylase	Bacterial reverse mutation assay (Ames)	Not mutagenic
		In vitro Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes	Not clastogenic

		90-day Oral Gavage Study in Rats	No adverse effects
XVI. *T. reesei* (heterologous rDNA)	Cellulase, beta-glucosidase, hemicellulase	Bacterial reverse mutation assay (Ames)	Not mutagenic
		In vitro Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes	Not clastogenic
		90-day Oral Gavage Study in Rats	No adverse effects
XVII. *T. reesei* (heterologous rDNA)	Glucoamylase	Bacterial reverse mutation assay (Ames)	Not mutagenic
		I*n vitro* chromosome assay, human lymphocytes	Not clastogenic
		90-day oral (gavage) toxicology study, rats	No adverse effects
XVIII. *T. reesei* (heterologous rDNA)	Hydrophobin	Bacterial reverse mutation assay (Ames)	Not mutagenic
		In vitro chromosomal aberration assay, human lymphocytes	Not clastogenic
		In vitro Mammalian cell mutation test	Not clastogenic
XIX. *T. reesei* (heterologous rDNA)	Catalase	Bacterial reverse mutation assay (Ames)	Not mutagenic
		In vitro chromosomal aberration assay, human lymphocytes	Not clastogenic
		Subchronic toxicity 90-day gavage in rats	No adverse effects
XX. *T. reesei* (heterologous rDNA)	Glucoamylase	Bacterial reverse mutation assay (Ames)	Not mutagenic
		In vitro chromosomal aberration assay, Human lymphocytes	Not clastogenic
		Subchronic toxicity 90-day gavage study in rats	No adverse effects
XXI. *T. reesei* (heterologous rDNA)	Xylanase I	Bacterial reverse mutation assay (Ames)	Not mutagenic
		In vitro chromosomal aberration assay, Human lymphocytes	Not clastogenic

		Subchronic 90-day subchronic oral toxicity study, rats	No adverse effects
XXII. *T. reesei* (heterologous rDNA)	Xylanase II	Bacterial reverse mutation assay (Ames)	Not mutagenic
		In vitro Mammalian Chromosomal Aberration Assay in Human Peripheral Blood Lymphocytes	Not clastogenic
		Repeated dose 90-day oral toxicity in rats	No adverse effects

Appendix 9: GRAS Panel Report

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Michael W. Pariza, Member

April 7, 2017

Vincent Sewalt, PhD
Senior Director, Product Stewardship & Regulatory
DuPont Industrial Biosciences
Danisco US, Inc.
925 Page Mill Road
Palo Alto, CA 94304

RE: GRAS opinion on the intended use as an α-glucosidase of DuPont's α-glucosidase-transglucosidase enzyme complex from *Aspergillus niger* that is expressed in a non-pathogenic, non-toxigenic strain of *Trichoderma reesei*

Dear Dr. Sewalt,

I have reviewed the information you provided on DuPont's α-glucosidase-transglucosidase enzyme preparation, designated αG-TrTG, which is expressed by *Trichoderma reesei* MorphTG#626 (GICC03289), a production strain that has been genetically modified to over-express a native carbohydrase enzyme complex from *Aspergillus niger* AGME9 that possesses both α-glucosidase and transglucosidase activities. The intended new use for αG-TrTG is as an α-glucosidase enzyme processing aid in the manufacture of lysine, organic acids (e.g., lactic, citric and succinic acids), and monosodium glutamate (MSG). Previously this identical αG-TrTG protein was described in GRN 315 for use as a transglucosidase enzyme processing aid in the conversion of malto-oligosaccharides in starch from grains to isomalto-oligosaccharides, and as one of the enzymes in an enzyme cocktail utilized in the production of potable alcohol from molasses. FDA responded to GRN 315 with a "no questions" letter dated May 10, 2010.

In evaluating this new use for αG-TrTG as an α-glucosidase processing aid, I considered the biology of *T. reesei* and *A. niger* and their histories of safe use in food-grade enzyme manufacture; safety evaluation studies on the αG-TrTG enzyme produced by *T. reesei* MorphTG#626 (GICC03289); safety evaluation studies on other food grade enzymes expressed by DuPont's safe lineage of *T. reesei* production strains; the information that you provided regarding the safe lineage of the production organism, cloning methodology, manufacturing

materials and procedures, and product specifications; and information available in the peer-reviewed scientific literature.

By way of background, *T. reesei* is used widely by enzyme manufacturers worldwide for the production of enzyme preparations that are, in turn, used in human food, animal feed, and numerous industrial enzyme applications. DuPont's lineage of safe *T. reesei* production strains, including *T. reesei* MorphTG#626 (GICC03289), was derived through a series of modifications from *T. reesei* QM6a, the original non-pathogenic and non-toxigenic wild-type parental strain used to produce this safe lineage of *T. reesei* enzyme production strains. Published literature, government laws and regulations, for example FR 64:28658-28362 (1999), reviews by expert panels such as FAO/WHO JECFA (1992), and DuPont's (legacy Genencor and Danisco) unpublished safety studies, all support the conclusion that the lineage to which these production strains belong is safe and suitable for use in the manufacture of food-grade and feed-grade enzymes.

Strains within this safe lineage are used to manufacture many food and feed enzymes, including chymosin, transglucosidase, cellulases, glucoamylase, α-amylase, β-glucosidase/cellulase, acid fungal protease, α-glucosidase, lipase, xylanase and phytase. The enzyme products from 19 production strains within this safe lineage, and in two cases the production strains themselves, have been subjected to toxicology testing and rigorous safety evaluation in accordance with the Pariza-Johnson decision tree (Regulatory Toxicol. Pharmacol. 33: 173-186,2001). Some of these enzymes are also the subject of GRAS notification documents that are listed on the FDA GRAS Notice Inventory, for example GRN 230, 315, 333, 372, and 567, all of which carry the decision statement, "FDA has no questions." GRN 315 is particularly relevant to this GRAS opinion in that it concerns the use of this same enzyme, αG-TrTG from *T. reesei* Morph TG#626 (GICC03289), for the production of isomalto-oligosaccharide syrups from starch, and potable ethanol from molasses.

Aspergillus niger is also widely used by enzyme manufacturers worldwide for the production of enzyme preparations for use in human food, animal feed, and numerous other industrial enzyme applications. The αG-TrTG enzyme gene that was cloned into the *T. reesei* MorphTG#626 (GICC03289) production strain was obtained from *A. niger AGME9*, a non-pathogenic and non-toxigenic strain that has a history of safe use in the manufacture of food-grade and feed-grade α-glucosidase-transglucosidase.

The native αG-TrTG from *A. niger AGME9* was evaluated for acute, genotoxic and subchronic toxicity. No dose-related adverse events were observed in any of these studies. The NOAEL for native αG-TrTG, calculated from the highest dose tested in a 90 day feeding study in Wistar rats, was 74.8 mg TOS/kg bw/day, equivalent to 63.64 mg total protein/kg bw/day. The safety of αG-TrTG enzyme produced by *T. reesei* MorphTG#626 (GICC03289) was also evaluated for acute, genotoxic and subchronic toxicity. No dose-related adverse events were observed in any of these studies. Daily administration by gavage of αG-TrTG produced by *T. reesei* MorphTG#626 (GICC03289) to Wistar rats for 13 weeks did not result in overt signs of systemic toxicity. Under the conditions of this assay, the NOAEL (no observed adverse effect level) was established at the highest dose tested, 63.64 mg total protein/kg bw/day corresponding to 74.8 mg TOS/kg bw/day.

Using the budget method, human consumer exposure to αG-TrTG expressed by *T. reesei* MorphTG#626 (GICC03289) from all intended uses (current uses and the new uses that are the subject of this opinion) is estimated to be 0.443 mg TOS/kg bw/day, which gives a margin of safety of 169. The margins of safety for the use of αG-TrTG produced by *T. reesei* MorphTG#626 (GICC03289) to manufacture lysine for addition to animal feeds intended for cattle, pigs or poultry, respectively, exceeds 2,000.

The cloning techniques and methodologies employed to construct *T. reesei* MorphTG#626 (GICC03289) are appropriate for use in the genetic modification of production strains for food and feed ingredient manufacture. In addition, the manufacturing process including the ingredients used for fermentation, extraction and concentration of αG-TrTG, and the specifications for αG-TrTG, are appropriate for food and feed ingredients.

Based on the foregoing, I concur with the evaluation made by DuPont that the *T. reesei* MorphTG#626 (GICC03289) production strain is safe and appropriate to use for the manufacture of food-grade and feed-grade αG-TrTG. I further conclude that the αG-TrTG enzyme, manufactured in a manner that is consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food-grade specifications, is GRAS (Generally Recognized As Safe) for use as α-glucosidase enzyme processing aid in the manufacture of lysine, organic acids (e.g., lactic, citric and succinic acids), and monosodium glutamate (MSG), in addition to the previous concurred uses as a transglucosidase enzyme processing aid in the conversion of malto-oligosaccharides in starch from grains to isomalto-oligosaccharides, and as one of the enzymes in an enzyme cocktail utilized in the production of potable alcohol from molasses.

It is my professional opinion that other qualified experts would also concur in this conclusion.

Please note that this is a professional opinion directed at safety considerations only and not an endorsement, warranty, or recommendation regarding the possible use of the subject product by you or others.

Sincerely,

(b) (6)

Michael W. Pariza
Member, Michael W. Pariza Consulting, LLC
Professor Emeritus, Food Science
Director Emeritus, Food Research Institute
University of Wisconsin-Madison

Pages 45-53 and 61-94 have been removed in accordance with copyright laws. The removed references are:

Sewalt, V, Shanahan, D, Gregg, L, La Marta, J, and Carrillo, R. 2016. The Generally Recognized as Safe (GRAS) Process for Industrial Microbial Enzymes. *Industrial Biotechnology.* 12(5): 295- 302.

Dugan, F.M. 1998. A Note on *Trichoderma reesei* and *T longibrachiatum,* United States Federation for Culture Collections Newsletter. 28(2): 1- 2. Enzyme Applications in Encyclopedia of Chemical Technology

Samuels, G.J., Ismaiel, A., Mulaw, T.B., Szakacs, G., Druzhinina, I.S., Kubicek, C.P. and Jaklitsch, W .M., 2012. The Longibrachiatum Clade of Trichoderma: a revision with new species. Fungal Diversity, 55(1), pp.77-l 08.