A Trehalase Enzyme

Preparation Derived from

Trichoderma reesei

Expressing the Trehalase Gene

From

Trichoderma reesei

Is Generally Recognized As Safe

For Use in Food Processing

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

August 18, 2017

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1. GENERAL INTRODUCTION, STATEMENT AND CERTIFICATION

In accordance with 21 C.F.R. §170. 225, Danisco US Inc. submits the GRAS Notice for trehalase produced by submerged fermentation of *Trichoderma reesei* carrying the gene encoding the trehalase enzyme from *Trichoderma reesei*.

The trehalase enzyme product is intended for use during the fermentation process to hydrolyze the α-glucosidic *O*-linkage of trehalose, a product of yeast metabolism. This hydrolysis releases initially equimolar amounts of α- and β-D-glucose, which will be used in fermentation to manufacture organic acids (*e.g.,* lactic, citric, and succinic acid), monosodium glutamate (MSG), and potable alcohol. In these applications trehalase will be used as a processing aid and will 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 principle enzyme activity is trehalose glucohydrolase. Other names used are trehalase, α, α-trehalase, etc., as described in Section 2.2.1 of this submission. For consistency, this enzyme will be presented by the shorter name trehalase throughout the dossier.

The enzyme hydrolyzes of the α-glucosidic *O*-linkage of trehalose, releasing initially equimolar amounts of α- and β-D-glucose.

The EC number of the enzyme is 3.2.1.28, and the CAS number is 9025-52-9.

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

Our safety evaluation agrees with the recent publication by the Enzyme Technical Association (Sewalt *et. al.*, 2016, see Appendix 1), which includes an evaluation of the production strain, the enzyme, and the manufacturing process (Part 6), as well as a determination of dietary exposure (Part 3). This generally recognized methodology, based on the decision tree by Pariza and Johnson (2001) and inclusive of published safety information, provides the common knowledge element of the GRAS status of trehalase enzyme notified to the FDA (Sewalt *et al.* 2017, see Appendix 1).

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 trehalase) 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; SCF, 1991; OECD,

1993; Berkowitz & Maryanski, 1989). The genetic modifications used to construct this production organism are well defined and 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 trehalase enzyme preparation from a genetically engineered strain of *T. reesei* expressing the trehalase enzyme from *T. reesei* is a Generally Recognized As Safe ("GRAS") substance for the intended food applications and is, therefore, exempt from the requirement for premarket 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 trehalase enzyme preparation is produced in a *Trichoderma reesei* strain expressing the gene encoding the trehalase from *Trichoderma reesei.*

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

The trehalase is intended to be used as a processing aid during the fermentation process to manufacture organic acids (*e.g.,* lactic-, citric-, and succinic acid), monosodium glutamate (MSG), and potable alcohol at 100 mg product/kg starch (equivalent to 36.72 mg TOS/kg starch).

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 Part 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 a complete, representative, and 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)

August 18, 2017

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

2.1 PRODUCTION ORGANISM

2.1.1 Production Strain

The production organism strain is a strain of *T. reesei* that has been genetically engineered to overexpress the trehalase gene from *T. reesei.*

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 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 trehalase gene under the regulation of the native *T. reesei* cellobiohydrolase (*cbh1*) promoter and the native *T. reesei* cellobiohydrolase (*cbh1*) transcription terminator, and the native *T. reesei* orotate phosphoribosyl transferase (*pyr2*) gene as a selectable marker. The inserted DNA was integrated into the recipient chromosome.

2.1.2 Recipient Organism

The host organism *T. reesei* strain RL-P37 was obtained from Dr. Bland S. Montenecourt. The derivation and characterization of strain RL-P37 has been published (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 *T. reesei* strain (QM6a). Strain QM6a is present in several public culture collections, such as the American Type Culture Collection as ATCC 13631. *T. reesei* has more recently been identified as a clonal derivative or anamorph of *Hypocrea jecorina* (Khuls *et al.*, 1996 and Dugan, 1998).

2.1.3 Trehalase Expression Plasmid

The genetic modification of the *T. reesei* host involved recombinant DNA techniques to introduce multiple copies of the gene encoding the wild type *T. reesei* trehalase into the *T. reesei* host.

The expression cassette comprised the native *T. reesei* cellobiohydrolase (*cbh1*) promoter, which was used to drive expression of the trehalase *trel* gene, the native trehalase gene *trel* from *T. reesei*, the native *T. reesei* cellobiohydrolase (*cbh1*) transcription terminator, and the native *T. reesei* orotate phosphoribosyl transferase (*pyr2*) gene as a selectable marker.

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 judged by trehalase 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

Classification	Trehalase
IUBMB Nomenclature	α, α-trehalase
IUBMB Number:	3.2.1.28
CAS Number:	9025-52-9
Reaction catalyzed:	Hydrolysis of the α-glucosidic *O*-linkage of trehalose, releasing initially equimolar amounts of α- and β-D-glucose.

2.2.2 Amino Acid Sequence

The amino acid sequence of the *T. reesei* trehalase is known and included in Appendix 2.

2.3 MANUFACTURING PROCESS

This section describes the manufacturing process for this trehalase enzyme which follows standard industry practice (Kroschwits, 1994; Aunstrup *et al.*, 1979; Aunstrup, 1979). For a diagram of the manufacturing process, see Appendix 3. 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 trehalase concentrate 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 (also known as defoamers) and flocculants used in the fermentation and recovery are used in accordance with cGMP per the September 11, 2003 FDA correspondence to ETA acknowledging the listed antifoams. Therefore, the maximum use level of these antifoams in the production process is <1.0%, cationic polymer flocculants < 1%, and anionic polymer flocculant at <0.025%.

Regarding potential major food allergens, glucose (which may be derived from wheat) will be used in the fermentation process and is consumed by the microorganism as nutrients. Therefore, 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 trehalase enzyme is manufactured by submerged fermentation of a pure culture of the genetically engineered strain of *T. reesei* described in Part 2. All equipments are 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.

2.3.3 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 30% dextrose, 4-7% sodium chloride, 0.26-0.35% sodium benzoate, and 0.09-0.12% potassium sorbate at pH 4.5-5.0. The remaining portion of the formulation is water.

The final trehalase 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:

Trehalase activity	9,000 U/g
Alpha, alpha-trehalase	10-15%
Dextrose	30%
Sodium Chloride	4-7%
Sodium benzoate	0.26-0.35%
Potassium sorbate	0.09-0.12%

The preparation includes TOS (total organic solids resulting from the fermentation) of approximately 36.72%.

2.4.2 Specifications

Trehalase preparation meets the purity specifications for enzyme preparations set forth in Food Chemicals Codex 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 4 verifying that meets FCC (2016) and JECFA (2006) specifications for enzyme preparations.

2.5 APPLICATION

2.5.1 Mode of Action

Trehalase catalyzes the hydrolysis of the α-glucosidic *O*-linkage of trehalose, releasing initially equimolar amounts of α- and β-D-glucose.

2.5.2 Use Levels

T. reesei trehalase preparation is intended for use during the fermentation process to convert trehalose, a product of yeast metabolism, to glucose, to serve as yeast-fermentable substrate. The fermentation targets include potable alcohol, organic acids such as lactic-, citric- and succinic acid, and monosodium glutamate (MSG). Hence, trehalase is used to maximize yield during the manufacture of these fermentation products.

In the above applications, the proposed application dose of trehalase is 100 mg trehalase product/kg starch fermentation substrate. The product contains 36.72% TOS.

2.5.3 Enzyme Residues in the Final Foods

The trehalase enzyme will be deactivated or removed during the subsequent production and refining processes for all applications. In the rare case that inactive trehalase enzyme is present in the processed food and is ingested; it will not be absorbed intact. Instead, the enzyme is expected to be broken down by the digestive system into small peptides and amino acids, with the latter being absorbed and metabolized, which is not expected to pose any human health risk.

3. DIETARY EXPOSURE

Trehalase will be used as a processing aid in the fermentation to convert trehalose, a product of yeast metabolism, to glucose. The fermentation targets include:

- Potable and fuel alcohol,
- Organic acids such as citric-, lactic- and succinic acid, and
- Monosodium glutamate, MSG

While we expect the trehalase to be not present in the final food or present as inactive residue in negligible amounts, the following conservative calculations assume that 100% of the enzyme remains in the processed food.

Analytical determination of the trehalase indicates that each ml of the enzyme preparation contains 36.72% TOS/mg.

The dose rate and process yield from starch for potable alcohol, organic acids, and amino acids and MSG are set to be the same; the exposure to trehalase via potable alcohol, organic acids, amino acid, and MSG is outlined below via the Budget Method (Hansen, 1966; Douglass *et al.*, 1997). This method has been used by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2001). The estimated yield of the potable alcohol, organic acids, amino acid, and MSG is at least 50%. Therefore, the concentration of TOS from trehalase in the fermentation products can be calculated, and it is summarized in the table below.

	Fermentation Products (potable alcohol, organic acids, and amino acids)
Dose (kg product/MT starch)	0.1
Dose (mg TOS/ kg starch)	36.72
Yield %	50
Concentration (mg TOS/kg product)	73.44

Estimation of daily consumption of trehalase from its potential uses in major commodities is based on the Budget method as supported by organizations such as the European Food Safety Authority (EFSA, 2011).

Liquid Foods

Potable alcohol manufacture was concluded not to be a worse-case scenario as the distillation process in potable alcohol manufacture denatures and precipitates enzyme protein and removes the vast majority of water-soluble substances from the alcohol. In addition, the maximum intake

of any alcoholic drink will be limited largely by the maximum intake of alcohol the body can tolerate, not by the volume of the drink. Hence the potential exposure to trehalase from the consumption of potable alcohol is negligible.

The worst-case concentration of TOS from fermentables in potable alcohol and ingredients used in liquid foods was determined to be 73.44 mg TOS/L (equivalent to 73.44 mg TOS/kg) from organic acids in beverages.

The concentration of organic acids (such as citric acid) in soft drinks is set at 0.13%. Therefore, a final concentration of TOS from trehalase in beverages can be calculated as shown in the table below.

	Organic acids (beverage)
Maximum Concentration (mg TOS /L fermentation product)	73.44
Ingredient concentration in beverages (%)	0.13
Enzyme TOS concentration in beverages (mg TOS /L)	0.10
Total TOS Concentration in beverages (mg TOS/L)	0.10

For selecting an overall maximum exposure via liquids, the worst-case TOS concentration from exposure to organic acid is assessed. Hence, the exposure from organic acid was used in our risk assessment to represent worst case scenario exposures via intake from beverages, with the assumption that 25% of all consumed beverages are manufactured from raw materials treated with the trehalase.

Solid Foods

The trehalase enzyme preparation is used in the manufacture of organic acids such as citric acid, which can be used in bakery and dairy applications, and MSG.

Considering the maximum application rates of 36.72 mg TOS/kg starch and the estimated yield of organic acids and MSG is at least 50%; the concentration of fermentation products in solid foods was derived to be 73.44 TOS mg/kg.

Therefore, a final concentration of TOS from trehalase in solid food can be calculated as shown in the table below, with the highest concentration in organic acid from bakery and dairy applications.

	Organic acid	MSG in powdered soup
Maximum Concentration (TOS mg/kg ingredient)	73.44	73.44
Ingredient concentrate in solid food (%)	2*	1**
Enzyme TOS concentration in solid food (TOS mg/kg solid food)	1.47	0.73

* http://www.hc-sc.gc.ca/fn-an/securit/addit/list/10-ph-eng.php (double the use rate restriction of citric acid in cocoa product as the worst case)
** http://www.hc-sc.gc.ca/fn-an/securit/addit/msg_qa-qr-eng.php (double the use rate in the restriction of the guideline as the worst case)

Hence, the exposures from organic acids were used in our risk assessment to represent worst-case scenario exposures via intake from solid food, with the assumption that 50% of all consumed solid foods are manufactured from raw materials treated with the trehalase.

HUMAN EXPOSURE ASSESSMENT

In this assessment, the Budget method is used. This method was previously used by JECFA (FAO/WHO, 2001) and contains 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 will be combined with the TMDI for beverages in the risk assessment.

- ***Estimation of the TMDI for Liquid Foods:***

Since exposure of trehalase from organic acid represents a worst-case scenario. To represent worst-case scenario exposures via intake of beverages, in which we assume that 25% of all consumed beverages are manufactured from raw materials treated with the trehalase. As presented above enzyme exposure from distillation is disregarded due to the effects of distillation on the enzyme (denature and precipitation) and the self-limiting consumption of distilled spirits.

Beverage (non-milk) intake	100	ml/kg bw/day
Processed beverage intake (25%)	25	ml/kg bw/day
Enzyme TOS in soft drinks via organic acid (worst case)	0.10	mg TOS/L
TMDI beverages	0.0025	mg TOS/kg bw/day

Estimation of the TMDI for Solid Foods

Organic acid application in baking and dairy is higher than the application of MSG in soup, which the rationale supporting use of organic acid applications to represent the worst case scenario.

Solid food intake	25	g/kg bw/day
Processed food treated with enzyme (50%)	12.5	g/kg bw/day
Enzyme TOS in solid food as worse case	1.47	mg TOS/kg final food
TMDI solid food	0.018	mg TOS/kg bw/day

The Theoretical Maximum Daily Intake (TMDI)- total

TMDI $_{\text{beverages}}$	0.0025	mg TOS/kg bw/day
TMDI $_{\text{Solid food}}$	0.018	mg TOS/kg bw/day
TMDI total	0.0205	mg TOS/kg bw/day

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, which is based on scientific procedures rather than 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, QM6a, 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 *Trichoderma longibrachiatum*. Later however, evidence emerged indicating that the two species are not identical (Meyer *et al.*, 1992; Dugan, 1998, see Appendix 5). 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 *T. reesei* and *H. jecorina* are in use in the scientific literature to refer to essentially the same microorganism species (Samuels *et al*, 2012, see Appendix 5). Unfortunately, the name *T. 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 QM9414 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* QM6a and its derivatives (including QM9414), in which the Agency acknowledged that under normal submerged fermentation conditions paracelsin is not produced. Strain QM9414 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 trehalase enzyme preparation, the subject of this submission, is *T. reesei* strain LVS-ETD #23, which was produced from strain RL-P37 using recombinant DNA methods. The purpose of this genetic modification is to express the trehalase from *T. reesei* in *T. reesei*. *T. reesei* RL-P37, a commercial production strain produced from several classical mutagenesis steps from the well-known wild-type strain QM6a. Virtually all *T. reesei* strains used all over the world for industrial cellulase production today are derived from QM6a. 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).[2]

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 trehalase. The strain is non-pathogenic and non-toxigenic.

6.1.2 Safety of the donor source

The donor strain used in construction of the microorganism as a source for the trehalase gene was the same as the host, therefore the conclusions of safety for the host also apply for the donor source.

6.2 SAFETY OF THE MANUFACTURING PROCESS

The manufacturing process to produce trehalase will be conducted in a manner like 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 FAO/WHO (JECFA, 2006).

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

[2] https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices

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 TREHALASE

6.3.1 Allergenicity

According to Pariza and Foster (Pariza and Foster 1983), there have been no confirmed reports of allergies in consumers caused by enzymes used in food processing.

In 1998 the Association of Manufacturers of Fermentation Enzyme Products (AMFEP, 1998) 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 the 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 trehalase 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. To conduct the bioinformatic analysis of subtilisin, three FASTA searches were performed: 1) a full length amino acid sequence search and 2) a sliding 80-amino acid window search and 3) an 8-amino acid search. Based on the sequence homology alone, it was concluded that the trehalase 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."

Trichoderma reesei trehalase (mature) sequence is given in Appendix 2. A full length amino acid sequence search with greater than 35% identity and an E-value of < 0.1 to known allergens using the Food Allergy Research and Resource Program (FARRP) on the AllergenOnline database[1] January 18, 2017 V17, which contains 1956 peer-reviewed allergen sequences[2] confirmed no hits.

There was also no match to allergens by identity across 80 amino acids exceeding 35%. FASTA alignment of the above sequence with known allergens also using the AllergenOnline database[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.

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 allergy concern.

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

In conclusion, based on the sequence homology alone, *T. reesei* trehalase is unlikely to pose a risk of food allergenicity.

6.3.2 Safety of Use in Food

As noted in the Safety section 6.1, *T. reesei*, and enzyme preparations derived there from, including cellulase, beta-glucanase, xylanase, and acid fungal protease enzyme preparations, are well recognized by qualified experts as being safe. Published literature, government laws and regulations, reviews by expert panels such as JECFA, as well as Danisco US Inc.'s own unpublished safety studies, support such a conclusion.

T. reesei is widely used by enzyme manufacturers around the world to produce enzyme preparations for use in human food, animal feed, and numerous industrial enzyme applications. It is a known safe host for enzyme production.

Further, trehalase is naturally present in the human intestines with the greatest concentration of trehalase activity noted in the small intestine (Asp *et al.*, 1975). Trehalase within the digestive systems has been observed in humans as early as 10-14 weeks after conception and found at adult concentrations at birth (Galand, 1989). Therefore, there is normal exposure to trehalase within the human digestive system.

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

1. **Is the production strain[1] genetically modified[2,3]?** Yes, go to 2.

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

[1] 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.
[2] 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.
[3] 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.

3a. Does the expressed enzyme product which is encoded by the introduced DNA[1,2] have a history of safe use in food[3]? Yes, trehalase from *T. reesei* has been a minor (undeclared) component of various food/feed enzyme preparations. Further, this glycosidase enzyme is similar in functionality to glucoamylase, which also has the same intended uses. Also, a literature search did not reveal any aspects of concern, and sequence blasts revealed no homology to known food allergens. Finally, trehalase is used in very low amounts (10-100 fold less than other glucosidases used in ethanol manufacture), thus not adding significantly to the overall exposure to glucosidase enzymes. Go to 3c.

3c. Is the test article free of transferable antibiotic resistance gene DNA[4]? Yes. Antibiotic resistance genes were not used in the construction of the production strain. 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 and free of unsafe attributes. 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. The inserted DNA is well characterized. The production strain does not produce toxic metabolites of concern as confirmed by T-2 toxin analysis. Go to 6.

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[1] 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 other sequences 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.

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

[3] 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.

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

6. **Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure[1]?** Yes. The *T. reesei* production strain pertains to the *T. reesei* safe strain lineage (Appendix 6). *T. reesei* safety as a production host and methods of modification are well documented and their safety has been confirmed through toxicology testing.

Conclusion: The test article is accepted.

6.3.3 Safety Studies

T. reesei trehalase is an enzyme preparation produced from *T. reesei* that can be used as a processing aid in the fermentation to manufacture organic acids *(e.g.,* lactic, citric, and succinic acids), monosodium glutamate (MSG), and potable alcohol.

Danisco US Inc. has determined by scientific procedures that this production organism *T. reesei* pertains to a safe strain lineage. A review of all toxicology studies conducted with enzyme preparations produced by different strains of Danisco US Inc.'s *T. reesei* (Appendix 6) indicates that, regardless of the production organism 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 in any of the 90-day oral toxicity studies performed on enzymes produced with members of this *T. reesei* lineage.

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 6).

Based on strain lineage, the production strain most closely related to the trehalase production strain, is strain *T. reesei* producing *Fusarium verticillioides* xylanase Toxicology studies with *F. verticillioides* xylanase from *T. reesei* have been conducted, and the data can be extrapolated to trehalase from *T. reesei.* This approach is in line with the Safe Strain Lineage concept (Pariza and

[1] 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

Johnson, 2001) endorsed by the enzyme industry and regulatory agencies. 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.:	20148091
Physical:	Fermentation liquid, brown
Enzyme activity:	109,759 NGXU/g
pH:	4.7
Specific gravity:	1.044 g/ml
Total protein:	167.13 mg/ml
TOS:	17.12 %

A. Bacterial Reverse Mutation Assay – Ames assay

BioReliance: Report No. AE10JK.507001.BTL; Dupont No. 21091-513; Final report dated January 28, 2015

a. Procedure:

The test article, *Fusarium verticillioides* xylanase was tested in the Bacterial Reverse Mutation Assay using *Salmonella typhimurium* tester strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* tester strain WP2 *uvr*A in the presence and absence of Aroclor-induced rat liver S9. The assay was performed in two phases using the treat and plate modification of the preincubation method except as noted below. The plate incorporation methodology was used only for the positive control, 2-aminoanthracene (2AA), with *E. coli* in the presence of S9 activation. The first phase, the initial toxicity-mutation assay, was used to establish the dose range for the confirmatory mutagenicity assay, and to provide a preliminary mutagenicity evaluation. The second phase, the confirmatory mutagenicity assay, was used to evaluate and confirm the mutagenic potential of the test article. Dosing formulations were adjusted for total protein content based on the concentration as supplied at 167.97 mg/mL.

In the treat and plate method, the volumes of S9 mix, Sham mix, bacteria and test article, vehicle or positive control were increased by a factor of 2.5 or 3.5 to ensure sufficient volume of resuspended bacteria to plate the desired number of replicates. Water was selected as the solvent of choice based on information provided by the Sponsor and compatibility with the target cells.
In the initial toxicity-mutation assay, the maximum dose tested was 5000 µg per plate; this dose was achieved by diluting the test article at the Sponsor-provided concentration of 167.97 mg/mL to a concentration of 100 mg/mL and using a 50 µL plating aliquot. The dose levels tested were

1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate. The test article formed clear solutions in sterile water for injection-quality, cell culture grade water (hereafter referred to as sterile water) from 0.030 to 100 mg/mL. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation. Neither precipitate nor toxicity was observed. Based on the findings of the initial toxicity-mutation assay, the maximum dose plated in the confirmatory mutagenicity assay was 5000 µg per plate.

In the confirmatory mutagenicity assay, no positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation. The dose levels tested were 50, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed.

b. Results:

The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, the test article did not exhibit any mutagenic responses in either the presence or absence of Aroclor-induced rat liver S9. Therefore, the test article was concluded to be negative in this assay.

c. Evaluation

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, did not cause a positive mutagenic response with any of the tester strains in either the presence or absence of Aroclor-induced rat liver S9. Therefore, the test article was concluded to be negative in this assay.

B. In vitro Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes

BioReliance, Report No. AE10JK.341.BTL; Dupont No. 21091-544; Final report dated April 03, 2015

a. Procedure

The purpose of this study was to evaluate the potential of a test article and/or its metabolites to induce structural chromosomal aberrations in Human Peripheral Blood Lymphocytes (HPBL) in the presence and absence of an exogenous metabolic activation system (Aroclor-induced rat liver S9). A preliminary toxicity test was performed to establish the dose range for testing in the cytogenetic test. The chromosome aberration assay was used to evaluate the clastogenic potential of the test article. In both assays, HPBL cells were treated for 4 and 20 hours in the non-activated test system and for 4 hours in the S9-activated test system. All cells were harvested 20 hours after

treatment initiation. Dosing formulations were adjusted for total protein content based on the concentration as supplied at 167.97 mg/mL. Water was used as a vehicle.

b. Results

In the preliminary toxicity assay, the doses tested ranged from 0.5 to 5000 µg/mL. The test article was soluble in water and in the treatment medium at all concentrations tested at the beginning and conclusion of the treatment period. The osmolality in treatment medium of the highest dose level tested, 5000 µg/mL, was 266 mmol/kg. The osmolality of the vehicle (water) in the treatment medium was 257 mmol/kg. The osmolality of the test article dose level in treatment medium is acceptable because it did not exceed the osmolality of the vehicle by more than 120%. The pH of the highest dose level of test article in treatment medium was 7.5. Substantial toxicity (at least 50% reduction in mitotic index relative to the vehicle control) was not observed at any dose level in any of the treatment conditions. Based on these findings, the doses chosen for the chromosome aberration assay ranged from 1000 to 5000 µg/mL for all three treatment conditions.

In the chromosome aberration assay, the test article was soluble in water and in the treatment medium at all concentrations tested at the beginning and conclusion of the treatment period. The pH of the highest dose level of test article in treatment medium was 7.5. Substantial toxicity (at least 50% reduction in mitotic index relative to the vehicle control) was not observed at any dose level in any of the treatment conditions. Based on these findings, the doses chosen for microscopic analysis were 2500, 3500, and 5000 µg/mL for all three treatment conditions. The percentage of cells with structural or numerical aberrations in the test article-treated groups was not significantly increased relative to the vehicle control at any dose level ($p > 0.05$, Fisher's Exact test).

c. Evaluation

Under the conditions of the assay described in this test, *Fusarium verticillioides* xylanase was concluded to be negative for the induction of structural and numerical chromosome aberrations in both the non-activated and S9-activated test systems. *Fusarium verticillioides* xylanase was considered to be negative in the *In Vitro* Mammalian Chromosome Aberration Assay in HPBL.

C. 13-week Oral (Gavage) Toxicity Study in CD Rats

MPI Research, Inc.: Report No. 125-203; DuPont No. 21091-1026; Final report dated October 19, 2015

a. Procedure

The objective of this study was to investigate the potential toxicity of *Fusarium verticillioides* xylanase to induce systemic toxicity after repeated daily oral administration to Charles River CD rats of both sexes for 90 continuous days. Groups of 10 animals per sex were treated by oral gavage with 0 (deionized water), 250, 500, or 1000 mg TOS/kg bw/day. The dose volume was set at 10 mL/kg.

The animals were pair housed (same sex) in solid bottom cages with nonaromatic bedding in an environmentally controlled room. The animals were individually housed during times of functional observational battery (FOB) data collection and urine collection for clinical pathology analysis. Block Lab Diet® (Certified Rodent Diet #5002, PMI Nutrition International, Inc.) was available *ad libitum,* except during designated periods. The lot number from each diet lot used for this study was recorded. Certification analysis of each diet lot was performed by the manufacturer. Tap water was available *ad libitum* via an automatic watering system. All groups were housed under controlled temperature, humidity, and lighting conditions.

All animals were observed for morbidity, mortality, injury, and the availability of food and water twice daily. Assessments of neurobehavioral effects and general toxicity were based on mortality, functional observational battery (FOB) evaluations, locomotor activity, clinical observations (including cageside clinical observations), body weight, and food consumption (including food efficiency); ophthalmoscopic examinations; and clinical and anatomic pathology.

b. Results

No test article-related effects were reported among clinical observations, ophthalmic observations, body weight measurements, food consumption or food efficiency values, functional observation battery tests, locomotor activity evaluations, hematology, coagulation, clinical chemistry, or urinalysis parameters, or organ weight, macroscopic, or microscopic pathology findings.

c. Evaluation and conclusion

Male and female CD® [Crl:CD(SD)] rats were dosed with *Fusarium verticillioides* xylanase at 0, 250, 500 or 1000 mg TOS/kg bw/day daily for 90 days. No adverse test article related findings were observed. Under the conditions of this study, the no-observed-adverse-effect-level (NOAEL) is the high dose level, 1000 mg TOS/kg bw/day. This NOAEL is equivalent to approximately 967.8 mg protein/kg bw/day.

6.4 OVERALL SAFETY ASSESSMENT

6.4.1 Identification of the NOAEL

In the 90-day oral (gavage) study in rats, a NOAEL was established at 1000 mg Total Organic Solids (TOS) /kg bw/day equivalent to 967.8 mg Total Protein/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 *Fusarium verticillioides* xylanase is through oral ingestion, selection of this NOAEL is thus appropriate.

NOAEL: 1,000 mg TOS/kg bw/day = 967.8 mg TP/kg bw/day

6.4.2 Conclusion

Determination of the margin of safety

The margin of safety is calculated by dividing the NOAEL obtained from the 90-day oral (gavage) study in rats by the human exposure (worst-case scenario) assessed in Part 3. If the margin of safety is greater than 100, it suggests that the available toxicology data support the proposed uses and application rates.

$$\text{Margin of Safety} = \frac{\text{No Observed Adverse Effect Level (NOAEL)}}{\text{Maximum Daily Exposure}}$$

$$\text{Margin of Safety} = \frac{1{,}000 \text{ mg TOS/kg bw/day}}{0.0205 \text{ mg TOS/kg bw/day}}$$

Margin of Safety = 48780

6.5 BASIS FOR GENERAL RECOGNITION OF SAFETY

As noted in the Safety sections above, *T. 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 Danisco US Inc.'s (operating as DuPont Industrial Biosciences) own unpublished safety studies, support such a conclusion.

T. 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 Danisco US Inc. has been demonstrated to be safe.

The exposure of trehalase from *T. reesei* as a food processing aid in the fermentation to manufacture organic acids (*e.g.,* lactic, citric, and succinic acid), monosodium glutamate (MSG), and potable alcohol is assessed based on a battery of toxicology studies conducted with AfGA glucoamylase from *T. reesei.* This extrapolation of toxicology information is in keeping with the safe strain lineage concept of Pariza and Johnson (2001).

Genotoxicity assays were conducted with *Fusarium verticillioides* xylanase and under the conditions of these assays *Fusarium verticillioides* xylanase is not classified as a mutagen, a clastogen or an aneugen. The systemic toxicity of *Fusarium verticillioides* xylanase was investigated in an oral study (90-day) and daily administration of *Fusarium verticillioides* xylanase for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at 1,000 mg TOS/kg bw/day.

Based on a worst-case scenario that a person is consuming trehalase from organic acids, amino acids, MSG, and potable alcohol containing trehalase, the cumulative daily exposure of 0.0205 mg TOS/kg bw/day.

Based on a margin of safety (48780) far greater than 100 even in the worst-case, the proposed uses of trehalase in organic acids, amino acids, MSG, and potable alcohol are not a human health concern and are supported by existing toxicology data.

Based on the publicly available scientific data from the literature and additional supporting data generated by Danisco US Inc. (operating as DuPont Industrial Biosciences), and the decision tree analysis using generally recognized evaluation methodology (Pariza and Johnson, 2001; Sewalt *et al.*, 2016), the company has concluded that trehalase from *T. reesei* strain is safe and suitable for use as processing aid in the fermentation to manufacture organic acids (*e.g.,* lactic, citric, and succinic acid), monosodium glutamate (MSG), and potable alcohol. Collectively, the use of published information and evaluation methods provide a strong common knowledge element, based upon which this trehalase can be considered Generally Recognized as Safe (GRAS) for its intended uses. 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 7).

7. SUPPORTING DATA AND INFORMATION

7.1 LIST OF THE APPENDIXES

Appendix 1: ETA Published Paper on GRAS for Microbial Enzymes by Sewalt *et. al.* (2016) and Letter to the Editor by Sewalt *et al.* (2017)

Appendix 2: The Amino Acid Sequence of the trehalase

Appendix 3: The Manufacturing Process

Appendix 4: Certificate of Analysis (3 lots)

Appendix 5: *Trichoderma reesei* taxonomy articles

Appendix 6: *Trichoderma reesei* Strain Lineage and Summary of Safety Studies

Appendix 7: External Expert Opinion Letter from Dr. Michael Pariza

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Appendix 1: ETA Published Paper on GRAS for Microbial Enzymes by Sewalt *et. al.* (2016) and Letter to the Editor by Sewalt *et al.* (2017)

Pages 36-46 and 53-86 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. *Industr. Biotechnol.* 12: 295-302. https://doi.org/10.1089/ind.2016.0011 .

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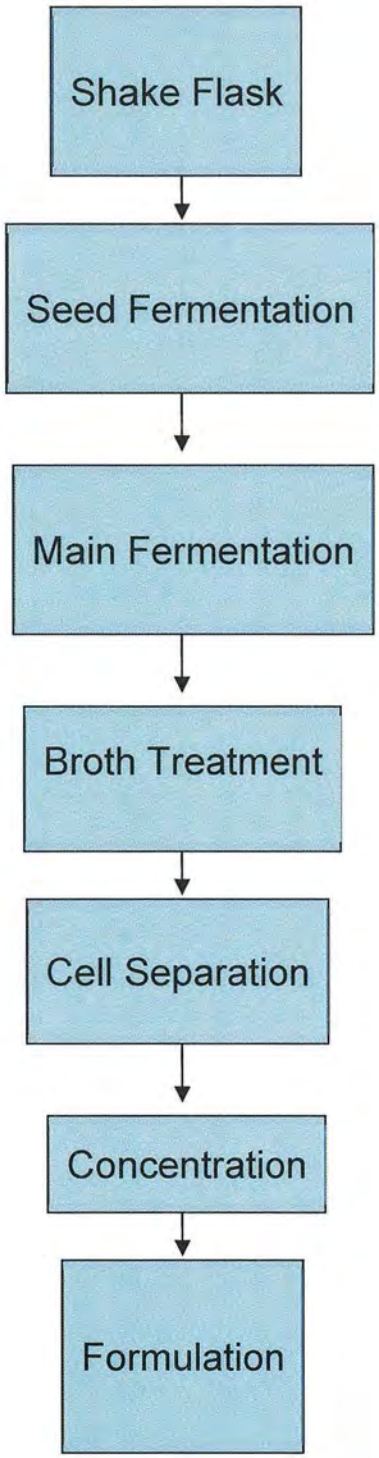
Appendix 3: Manufacturing Process of Trehalase



Appendix 4: Certificate of Analysis (3 lots)

CERTIFICATE OF ANALYSIS

PRODUCT: **OPTIMASH® TREHALASE**

BATCH: **1682695890**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITIES			
Trehalase	THU/g	9000 min.	11251
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

This product complies with the FAO/WHO and Food Chemicals Codex recommended specifications for food grade enzymes and contains permitted levels of stabilizers and preservatives.

6-Jun-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: **TRH-Trehalase Formulated Concentrate**

BATCH: **1682865745**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITIES			
Trehalase	THU/g	10000-14000	11717
PHYSICAL PROPERTIES			
pH		4.0-5.0	4.1
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

This product complies with the FAO/WHO and Food Chemicals Codex recommended specifications for food grade enzymes and contains permitted levels of stabilizers and preservatives.

29-Jun-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: **OPTIMASH® TREHALASE**

BATCH: **1682865667**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITIES			
Trehalase	THU/g	9000 min.	11248
PHYSICAL PROPERTIES			
pH		4.0-5.0	4.1
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

This product complies with the FAO/WHO and Food Chemicals Codex recommended specifications for food grade enzymes and contains permitted levels of stabilizers and preservatives.

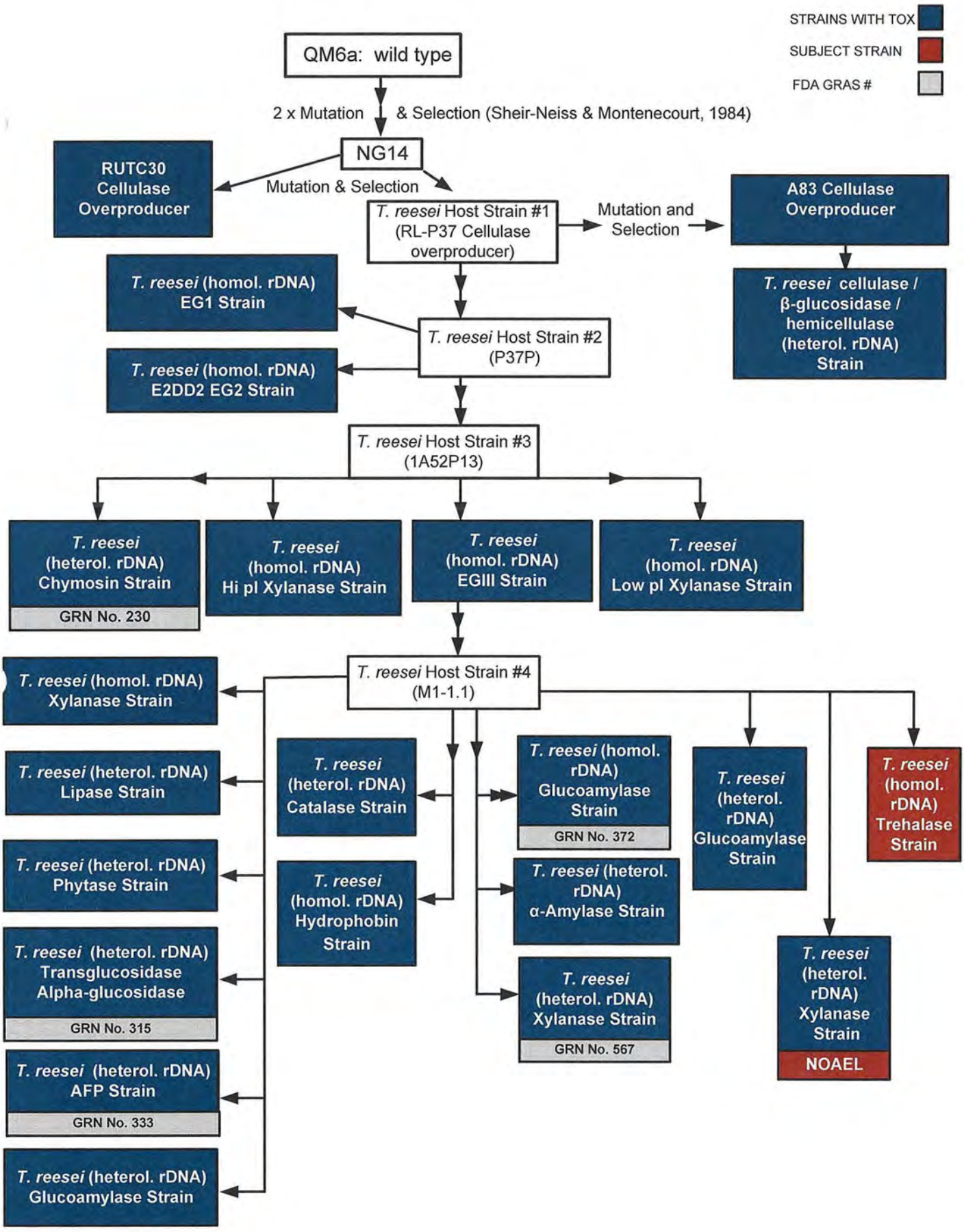
16-Jun-2017
Date

Kelly A. Altman
Manager, Quality Assurance

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

Appendix 5: *Trichoderma reesei* Taxonomy Articles

Appendix 6: *Trichoderma reesei* Strain Lineage and Summary of Safety Studies


STRAINS WITH TOX
SUBJECT STRAIN
FDA GRAS #
QM6a: wild type
2 x Mutation
& Selection (Sheir-Neiss & Montenecourt, 1984)
NG14
RUTC30 Cellulase Overproducer
Mutation & Selection
T. reesei Host Strain #1 (RL-P37 Cellulase overproducer)
Mutation and Selection
A83 Cellulase Overproducer
T. reesei cellulase / β-glucosidase / hemicellulase (heterol. rDNA) Strain
T. reesei (homol. rDNA) EG1 Strain
T. reesei Host Strain #2 (P37P)
T. reesei (homol. rDNA) E2DD2 EG2 Strain
T. reesei Host Strain #3 (1A52P13)
T. reesei (heterol. rDNA) Chymosin Strain
GRN No. 230
T. reesei (homol. rDNA) Hi pI Xylanase Strain
T. reesei (homol. rDNA) EGIII Strain
T. reesei (homol. rDNA) Low pI Xylanase Strain
T. reesei Host Strain #4 (M1-1.1)
T. reesei (homol. rDNA) Xylanase Strain
T. reesei (heterol. rDNA) Lipase Strain
T. reesei (heterol. rDNA) Phytase Strain
T. reesei (heterol. rDNA) Transglucosidase Alpha-glucosidase
GRN No. 315
T. reesei (heterol. rDNA) AFP Strain
GRN No. 333
T. reesei (heterol. rDNA) Glucoamylase Strain
T. reesei (heterol. rDNA) Catalase Strain
T. reesei (homol. rDNA) Hydrophobin Strain
T. reesei (homol. rDNA) Glucoamylase Strain
GRN No. 372
T. reesei (heterol. rDNA) α-Amylase Strain
T. reesei (heterol. rDNA) Xylanase Strain
GRN No. 567
T. reesei (heterol. rDNA) Glucoamylase Strain
T. reesei (homol. rDNA) Trehalase Strain
T. reesei (heterol. rDNA) Xylanase Strain
NOAEL

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 gray horizontal banners indicating the GRAS Notice number. The subject strain of this submission is the **Trehalase** producing strain indicated by the red color. The safety of the **Trehalase** enzyme is fully supported by repeated testing of other enzymes produced by members of this Safe Strain Lineage. The blue colored boxes indicate strains for which we conducted toxicology studies. The NOAEL for the **Trehalase** from the closely related production strain (labeled with a "NOAEL flag") is used to support the safety 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 Chinese Hamster	Not clastogenic

		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 Performed with Human	Not clastogenic

		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*	**Xylanase II**	Bacterial reverse	Not mutagenic

(heterologous rDNA)		mutation assay (Ames)	
		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 7: External Expert Opinion Letter from Dr. Michael Pariza

Michael W. Pariza Consulting LLC
7102 Valhalla Trail
Madison, WI 53719
(608) 271-5169
mwpariza@gmail.com

Michael W. Pariza, Member

August 16, 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 uses of DuPont's *Trichoderma reesei* Acid Trehalase produced by *T. reesei* LVS-ETD #23 (GICC20006118)

Dear Dr. Sewalt,

I have reviewed the information that you provided on DuPont's (legacy Genencor/Danisco) *Trichoderma reesei* acid trehalase enzyme preparation, which is produced by an improved and self-cloned strain, *T. reesei* LVS-ETD #23 (GICC# 20006118). The intended uses of this *T. reesei* acid trehalase enzyme preparation are in yeast fermentations to manufacture organic acids (i.e. lactic, citric, and succinic acids), amino acids (i.e. lysine), monosodium glutamate (MSG), potable alcohol, and fuel ethanol with resulting grain co-products destined for animal feed, where the enzyme is either not present in the final food or present as inactive protein in insignificant quantities having no function or technical effect in the final food.

In evaluating DuPont's *T. reesei* acid trehalase enzyme preparation, I considered the biology of *Trichoderma reesei*, relevant information available in the peer-reviewed scientific literature, and information that you provided regarding the cloning methodology that was utilized, the safe lineage of the production organism, and results of the safety evaluation studies that DuPont performed.

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* LVS-ETD #23 (GICC# 20006118), 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 Nevada1 (hemi) cellulase enzyme complex, chymosin, transglucosidase, cellulases, glucoamylase, α-amylase, β-glucosidase/cellulase, acid fungal protease, α-glucosidase, lipase, phytase, and xylanase. The enzyme products from 20 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 (MW Pariza and EA Johnson. *Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century*, Regulatory Toxicology and Pharmacology 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."

The *T. reesei* acid trehalase enzyme produced by *T. reesei* LVS-ETD #23 (GICC# 20006118) is native to this microbial species, and therefore present as a minor component in other enzyme preparations that are derived from *T. reesei,* including those within this lineage that have been previously subjected to rigorous safety evaluations. The DuPont production strain most closely related to *T. reesei* LVS-ETD #23 (GICC20006118) is *T. reesei* LVS-ETD FveXyn4-CL8-D3#15.2.3 (GICC03452), which produces NGX xylanase. NGX xylanase is similar in functionality to acid trehalase and has undergone rigorous safety evaluation as described below.

The safety of the NGX xylanase enzyme produced by *T. reesei* LVS-ETD-FveXyn4-CL8-D3#15.2.3 (GICC03452) was evaluated with a battery of standard toxicological tests that included sub-chronic (90-day) and acute oral toxicity studies in male and female Charles River rats as well as tests for the induction of dermal sensitization in mice, dermal and eye irritation in rabbits, bacterial mutagenesis (Ames test), and chromosomal aberrations in human lymphocytes. No treatment-related adverse effects were observed. In the subchronic rat gavage study the NOAEL was highest dose tested, 1000 mg TOS/kg bw/day, equivalent to 967.8 mg protein/kg bw/day. Extrapolating these data to the native *T. reesei* acid trehalase produced by *T. reesei* LVS-ETD #23 (GICC#20006118), DuPont estimates the margin of safety from all food applications of *T. reesei* acid trehalase to be 48780 for human consumers. In animal feed applications, the margins of safety are estimated to 504 for cattle, 403 for pigs, and 466 for poultry.

DuPont conducted a literature search using SciFinder (combined CAS and Medline databases,

on file with IB PS&R) with the search terms "trehalase" in combination with terms "toxicity" or "food safety". A review of the resulting publication abstracts revealed no indication that either acid or neutral trehalase are associated with toxicity or other adverse effects in humans or animals. Allergenic potential was assessed by comparing the *T. reesei* acid trehelase protein sequence with sequences of known allergenic proteins. The results of this analysis indicated that *T. reesei* acid trehalase is unlikely to pose an allergenic risk.

The safety of the *T. reesei* acid trehalase enzyme preparation produced by *T. reesei* LVS-ETD #23 (GICC# 20006118) was formally evaluated using the Pariza-Johnson decision tree. The conclusion of this analysis was that the test article (*T. reesei* acid trehalase enzyme preparation) was accepted.

DuPont has developed manufacturing conditions and specifications for enzyme manufacture, including the *T. reesei* trehalase enzyme preparation, that are appropriate and suitable for the manufacture of food-grade and feed-grade ingredients.

Based on the foregoing, I concur with DuPont's evaluation that the *T. reesei* LVS-ETD #23 (GICC# 20006118) production strain is safe to use for the manufacture of food-grade and feed-grade trehalase. I further concur that the DuPont *T. reesei* acid trehalase enzyme preparation, manufactured consistent with cGMP and meeting food grade specifications, is Generally Recognized As Safe (GRAS), based on scientific procedures, for use in yeast fermentation to manufacture organic acids (i.e. lactic, citric, and succinic acids), amino acids (i.e. lysine), monosodium glutamate (MSG), potable alcohol, and fuel ethanol with resulting grain co-products destined for animal feed, where the enzyme is either not present in the final food or present as inactive protein in insignificant quantities having no function or technical effect in the final food.

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

Highbarger, Lane A

From: Sewalt, Vincent <Vincent.Sewalt@dupont.com>
Sent: Monday, December 11, 2017 4:48 PM
To: Highbarger, Lane A
Subject: RE: GRN 000727 - Trehalase

Dear Lane,

The MW of the mature trehalase enzyme protein is predicted (based on its sequence) at 114 kDa.

We do run a protein gel as part of our internal qualifying process for new enzymes, not only to confirm the size/weight of the enzyme protein, also to make sure we have a consistent manufacture process, batch-to-batch.

The protein gel confirmed the MW of approximately 114 kDa.

Best regards
Vince

From: Highbarger, Lane A [mailto:Lane.Highbarger@fda.hhs.gov]
Sent: Monday, December 11, 2017 10:43 AM
To: Sewalt, Vincent <Vincent.Sewalt@dupont.com>
Subject: [EXTERNAL] GRN 000727 - Trehalase

Vince,

Did you run a gel to determine the MW of your trehalase enzyme, or any other method, and if so, what MW would you state that it is? I ask simply for completeness of the admin. record. All that I see in the notice is the AA sequence.

Thank you.

Lane A. Highbarger, Ph.D.
Consumer Safety Officer
U.S. Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
Division of Biotechnology and GRAS Notice Review
(w) – 240-402-1204

http://www.DuPont.com/corp/email_disclaimer.html