A Lysophospholipase Enzyme

Preparation Derived from

Trichoderma reesei

Expressing the Lysophospholipase Gene

From

Aspergillus niger

Is Generally Recognized As Safe

For Use in Food Processing

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

December 17, 2019

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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 lysophospholipase produced by submerged fermentation of *Trichoderma reesei* carrying the gene encoding the lysophospholipase enzyme from *Aspergillus niger*.

The lysophospholipase enzyme is intended for use to catalyze the hydrolysis of 2-lysophosphatidylcholine to form glycerophosphocholine and carboxylate. The lysophospholipase enzyme will be used in carbohydrate processing. In these applications, the lysophospholipase 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 accepted name of this enzyme is lysophospholipase. The systematic name of the principle enzyme activity is 2-lysophosphatidylcholine acylhydrolase. The IUBMB nomenclature is 2-lysophosphatidylcholine acylhydrolase. Other names used are lecithinase B, lysolecithinase; phospholipase B, lysophosphatidase, lecitholipase, phosphatidase B, lysophosphatidylcholine hydrolase, lysophospholipase A1, lysophopholipase L2, lysophospholipase-transacylase; neuropathy target esterase, NTE, NTE-LysoPLA, NTE-lysophospholipase, *etc*., as described in Section 2.2.1 of this submission. For consistency, this enzyme will be presented by the name "AnLPL" throughout the dossier.

The enzyme hydrolyzes 2-lysophosphatidylcholine to release glycerophosphocholine and carboxylate.

The EC number of the enzyme is 3.1.1.5, and the CAS number is 9001-85-8.

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

Our safety evaluation is consistent with the recent publication by the Enzyme Technical Association (Sewalt *et. al*., 2016),[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 this lipase enzyme notified to the FDA (Sewalt *et al.*, 2017).[2]

[1] https://doi.org/10.1089/ind.2016.0011
[2] http://www.enzymeassociation.org/?p=595

The safety of the production organism is considered to 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*) is discussed in Part 2 and 6 of this submission. The other 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 § 170.225 (c)(2) Name and Address of Notifier

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

1.2 § 170.225 (c)(3) Common or Usual Name of Substance

The lysophospholipase enzyme preparation is produced by a *Trichoderma reesei* strain expressing the gene encoding the lysophospholipase from *Aspergillus niger*.

1.3 § 170.225 (c)(4) Applicable Conditions of Use

The lysophospholipase is intended to be used as a processing aid in carbohydrate processing at 24.16 mg TOS/kg RM (raw material).

1.4 §170.225 (c)(5) Basis for GRAS Determination

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

1.5 §170.225 (c)(6) Exemption from Pre-market Approval

Pursuant to the regulatory and scientific procedures established in 21 C.F.R. §170.325, Danisco US Inc. has determined that its lysophospholipase enzyme preparation from a genetically

engineered strain of *T. reesei* expressing the lysophospholipase enzyme from *A. niger* is a Generally Recognized As Safe ("GRAS") substance for the intended food applications and is, therefore, exempt from the requirement for premarket approval.

1.6 §170.225 (c)(7) 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 can be sent to the Food and Drug Administration upon request.

1.7 §170.225 (c)(8) and (c)(9) 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.

GRN
Aspergillus niger Lysophospholipase in *Trichoderma reesei*
Danisco US, Inc. (Operating as DuPont Nutrition & Biosciences)

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.

[redacted]

12-17-2019

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Date

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

2.1 PRODUCTION ORGANISM

2.1.1 Production Strain

The production organism is a strain of *T. reesei* that has been genetically engineered to express the lysophospholipase gene from *A. niger*.

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 suitable for Good Industrial Large-Scale Practice (GILSP) worldwide. It also meets the criteria for a safe production microorganism as described by Pariza and Foster (1983). The production strain contains the *A. niger* lysophospholipase gene regulated under the expression signals of the endogenous *Trichoderma reesei cbh1* gene, and a copy of the expression cassette were integrated into the recipient chromosome using the *T. reesei pyr2* gene (orotate phosphoribosyl transferase) as selectable marker.

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 Lysophospholipase Expression Plasmid

The genetic modification of the *T. reesei* host involved recombinant DNA techniques to introduce a synthetic codon optimized gene encoding the wild type *A. niger* lysophospholipase into the *T. reesei* host.

The expression cassette comprised:

- Native *T. reesei cbh1* (cellobiohydrolase) gene promoter
- *Aspergillus niger* lysophospholipase gene
- Native *T. reesei cbh*I terminator
- *T. reesei pyr2* gene (orotate phosphoribosyl transferase) used as a selectable marker.

The inserted DNA was integrated into the recipient chromosome.

All these modifications were performed in such a way that no bacterial vector DNA remains present in the strain. No antibiotic resistance markers were inserted into the new microorganism. 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, PCR analyses, and genome sequencing 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 introduced lysophospholipase gene in the production strain proved to be completely stable after industrial scale fermentation as judged by lysophospholipase 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.[1]

[1] https://ac.els-cdn.com/S0273230005800807/1-s2.0-S0273230005800807-main.pdf?_tid=c89f62ce-5402-4e18-a3be-68ddbf116b10&acdnat=1530898844_165c4c45e811723d34f8db3e1878c745

2.2 ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

2.2.1 Enzyme Identity

Classification	Lysophospholipase
IUBMB Nomenclature	2-lysophosphatidylcholine acylhydrolase
IUBMB Number	3.1.1.5
CAS Number	9001-85-8
Reaction catalyzed	2-lysophosphatidylcholine + H_2O = glycerophosphocholine + a carboxylate

2.2.2 Amino Acid Sequence

The amino acid sequence of the *A. niger* lysophospholipase is known and included in Appendix 1, which is 100% identical to lysophospholipase 1 from *Aspergillus niger* CBS 513.88 and 99.36% identical to lysophospholipase from *Aspergillus awamori*. The molecular weight is 67.14 kDa.

2.3 MANUFACTURING PROCESS

This section describes the manufacturing process for this AnLPL lysophospholipase enzyme which follows standard industry practice (Kroschwitz, 1994; Aunstrup *et al*., 1979; Aunstrup, 1979). For a diagram of the manufacturing process, see Appendix 2. 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. §110.

2.3.1 Raw Materials

The raw materials used in the fermentation and recovery process for this AnLPL lysophospholipase concentrate are standard ingredients used in the enzyme industry (Kroschwitz, 1994; Aunstrup, 1979 and Aunstrup *et al*., 1979). All the raw materials conform to the specifications of the Food Chemicals Codex, 11th edition, 2018 ("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 antifoams (also known as defoamers) used in the fermentation and recovery are used in accordance with cGMP per the Food and Drug Administration (FDA) correspondence to Enzyme

Technical Association (ETA) acknowledging the listed antifoams and flocculants dated September 11, 2003.

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. No other major allergen substances will be used in the fermentation, recovery processes, or formulation of this product.

2.3.2 Fermentation Process

The AnLPL lysophospholipase 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.

2.3.3 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% glycerol, 5.5% sodium chloride, 0.35% sodium benzoate, and 0.2% potassium sorbate. The remaining portion of the formulation is water.

The final AnLPL lysophospholipase liquid concentrate 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 FCC, 11th edition (USP, 2018). 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 for the commercialized product:

- Enzyme 0.5% (w/w)
- Glycerol 30% (w/w)
- Sodium chloride 5.5% (w/w)
- Sodium benzoate 0.35% (w/w)
- Potassium sorbate 0.20% (w/w)
- pH 4.7-5.2

The remainder is water.

The preparation includes TOS (total organic solids resulting from fermentation), which is approximately 24.16% of the liquid concentrate.

2.4.2 Specifications

As mentioned, AnLPL lysophospholipase preparation meets the purity specifications for enzyme preparations set forth in FCC, 11th edition (USP, 2018). In addition, it also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by JECFA (2006).

The results of analytical testing of the 3 lots of product is given in Appendix 3 verifying that it meets USP (2018) and JECFA (2006) specifications for enzyme preparations.

2.5 APPLICATION

2.5.1 Mode of Action

Lysophospholipase catalyzes the following reaction.

2-lysophosphatidylcholine + H_2O = glycerophosphocholine + a carboxylate

2.5.2 Use Levels

The AnLPL lysophospholipase preparation is intended for use in in the carbohydrate processing including high fructose corn syrup to be used in food at 100 g enzyme product/metric ton dry material which is equivalent to 24.16 mg TOS/kg dry material in accordance with the principles of current Good Manufacturing Practice (GMP).

The average TOS content in product is 24.16 %.

2.5.3 Enzyme Residues in the Final Foods

The AnLPL lysophospholipase enzyme will be deactivated or removed during the subsequent production and refining processes for all applications. In the rare case that inactive lysophospholipase 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

AnLPL lysophospholipase will be used as a processing aid in carbohydrate processing, including the manufacture of sweeteners such as high fructose corn syrup (HFCS) to be used in food.

While we expect the lysophospholipase 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, as total organic solids (TOS).

The exposure to AnLPL lysophospholipase via carbohydrate processing 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 method enables to calculate a Theoretical Maximum Daily Intake (TMDI) based on conservative assumptions regarding physiological requirements for energy from food and the energy density of food rather than on food consumption survey data.

The Budget Method was originally developed for determining food additive use limits and is known to result in conservative estimations of the daily intake. The Budget Method is based on the following assumed consumption of targeted important foodstuffs and beverages (for less

important foodstuffs, *e.g.,* snacks, lower consumption levels are assumed). The assumption is for Processed food (50% of total solid food) and for soft drinks (25% of total beverages).

Average consumption over the course of a lifetime/kg body weight/day	Total solid food (kg)	Total non-milk beverages (l)	Processed food (50% of total solid food) (kg)	Soft drinks (25% of total beverages) (l)
	0.025	0.1	**0.0125**	**0.025**

The recommended use level of the AnLPL lysophospholipase is given, based on the raw materials used in the food process. The calculation considers how much solid or liquid food is obtained per kg raw material, and it is assumed that all the TOS will end up in the final product. Therefore, the concentration of TOS from lysophospholipase in the applications can be calculated/summarized as in the table below:

	Liquid Food	Solid Food
Application	**Sugar syrups**	**Modified Starch Sugar Syrups**
Raw Material (RM)	**Starch**	**Starch**
Final Food (FF)	**Soft Drink**	**Bakery, Dairy**
Dose (mg TOS/ kg RM)	24.16	24.16
Yield (RM/FF)	0.12	0.05
Concentration (TOS mg/L, Final food)	2.90	1.21

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 carbohydrate processing is the sole liquid food application represented and the max dosage used in this application represents the worst case scenario, in which we assume that 25% of all consumed beverages are manufactured from raw materials treated with AnLPL lysophospholipase.

Beverage (non-milk) intake	100	ml/kg bw/day
Processed beverage intake (25%)	25	ml/kg bw/day
Enzyme TOS in beverage via carbohydrate processing as worst case	2.90	mg TOS/L beverage
TMDI liquid food	0.072	mg TOS/kg bw/day

Estimation of the TMDI for Solid Foods

Carbohydrate processing is the sole solid food application represented and the max dosage used in this application represents 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	1.21	mg TOS/kg final food
TMDI solid food	0.015	mg TOS/kg bw/day

The Theoretical Maximum Daily Intake (TMDI)- total

TMDI Liquid food	0.072	mg TOS/kg bw/day
TMDI Solid food	0.015	mg TOS/kg bw/day
TMDI total	0.087	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 use levels are limited by economical reasons as customers are unlikely to use more enzyme than is needed to achieve the technical effects in order 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 is recognized as 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 common 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). 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). 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 – 2018) 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 (*i.e.*., 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. Enzyme manufacturers still confirm that the industrial enzyme preparations do not to have antibiotic activity per the specifications recommended by JECFA (2006).

T. reesei has a long history of safe use in industrial scale enzyme production. The safety of this species as an industrial enzyme producer has been reviewed by Nevalainen *et al.* (1994), 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 reviewed in approval dossiers for 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, at least 18 enzymes produced in *T. reesei* have been notified to FDA/CFSAN as GRAS for their intended uses and received a "no questions" letter[1], of which seven were for enzymes produced by members of Danisco/DuPont's *T. reesei* Safe Strain Lineage.

The production organism of the AnLPL lysophospholipase enzyme preparation, the subject of this submission, is *T. reesei* strain LVS-ETD AnLPL4-169-C10, which was produced from strain RL-P37 using recombinant DNA methods. The purpose of this genetic modification is to express the lysophospholipase from *A. niger* in *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. Danisco US, Inc. (operating as DuPont Nutrition & Biosciences) 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-toxigenic 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. Seven 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, GRN 567, GRN 703, GRN 727, and GRN 808).[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 the lysophospholipase. The strain is non-pathogenic and non-toxigenic.

[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

6.1.2 Safety of the donor source

The source for the AnLPL lysophospholipase sequence is *Aspergillus niger* CBS 513.88, which has a long history of safe use and is itself widely used in biotechnology for the production of enzymes (Pel *et al.*, 2007).

However, no actual strain was used as donor. The nucleic acid sequence of the *A. niger* lysophospholipase gene was obtained from NCBI (Genbank accession: XM_001393405.2). The DNA sequence was codon optimized for expression in *T. reesei* without changing the translated amino acid sequence. Only the lysophospholipase sequence was inserted into the genome of *T. reesei* and no other 'donor' sequences and since this gene encodes for a safe enzyme, the status of the donor organism is basically irrelevant to the safety of the AnLPL lysophospholipase enzyme produced in *T. reesei*.

Regardless, *A. niger* 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 citric acid (21 CFR §173.280) and a series of food-grade carbohydrases, oxidoreductases, lipases, glucanotransferase, and proteases (Pariza and Johnson, 2001; Olempska-Beer, 2006; 21 CFR §173.120, and multiple GRAS Notices).

6.2 SAFETY OF THE MANUFACTURING PROCESS

The manufacturing process to produce the AnLPL lysophospholipase is conducted in a manner like other food and feed enzyme production processes. It consists of a pure-culture fermentation process, cell separation, concentration, and formulation. The process is conducted in accordance with the current food good manufacturing practice (cGMP) as set forth in 21 C.F.R. §110. The resultant product meets the purity specifications for enzyme preparations of the Food Chemicals Codex, 11th Edition (US Pharmacopeia, 2018) and the general specifications for enzyme preparations used in food processing proposed by FAO/WHO (JECFA, 2006).

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. No other major allergen substances will be used in the fermentation, recovery processes, or formulation of this product.

6.3 SAFETY OF ANLPL LYSOPHOSPHOLIPASE

Lysophospholipase is a common secondary activity found in amyloglucosidase preparations produced by *Aspergillus niger*. Almost all amyloglucosidase enzyme preparations contain a significant amount of lysophospholipase as a secondary enzyme activity. Thus, exposure to lysophospholipase as a component of *A. niger* amyloglucosidase enzyme preparations has occurred for over 50 years. Since the introduction of wheat as a major raw material source in starch processing, the amount of lysophospholipase activity, in commercially available amyloglucosidase preparations, has increased.

Lysophospholipase derived from *Aspergillus nishimurae* expressed in *Trichoderma reesei* was the subject of a FDA GRAS Notice (GRN 653), which resulted in a "no questions" letter.

Other countries have approved lysophospholipase preparations derived from *A. niger, e.g.*, France and Australia/New Zealand (see Australian Standard 1.3.3).

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 lysophospholipase 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 lysophospholipase 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."

The *Aspergillus niger* AnLPL (mature) sequence is given in Appendix 1. 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] February 10, 2019 V19 which contains 2129 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 in Codex Commission (2009), researched by Herman *et al*. (2009) and further elaborated by Ladics *et al.* (2011) and on AllergenOnline.org that there is no evidence that a short contiguous amino acid match will identify a protein that is likely to be cross-reactive and that could be missed by the conservative 80 amino acid match (35%), this database does allow for isolated

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

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.

In conclusion, based on the sequence homology alone, *A. niger* lysophospholipase 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, alpha-glucosidase, transglucosidase, trehalase 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 a conclusion of safety.

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.

In addition to the allergenicity assessment described above, the safety of this AnLLPL lysophospholipase 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.

3a. Does the expressed enzyme product which is encoded by the introduced DNA[4,5] have a history of safe use in food[6]? Yes, Lysophospholipase has been used for years in food processing. Lysophospholipase derived from *Aspergillus nishimurae* expressed in *Trichoderma reesei* was filed GRAS Notice as GRN 653 and FDA has issued "No Question" letter. Thirteen phospholipases have been notified to FDA and FDA issued "No Question" letters for GRAS Notices (GRAS Notices 142, 145, 183, 204, 212, 490, 524, 574, 651, 653, 689, 728, and 811).[7]

In addition, the enzyme will be inactivated in the food manufacture process. The safety of the enzyme in the intended uses was supported by 1) a thorough investigation of the AnLPL lysophosphlipase sequence, identifying no potential risk for food allergenicity, 2) the production strain pertains to *T. reesei* safe strain lineage. Go to 3c.

3c. Is the test article free of transferable antibiotic resistance gene DNA[8]? Yes. Antibiotic resistance genes were not used in the construction of the production strain. Go to 3e.

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[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.

[4] 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.

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

[6] 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.

[7] https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&sort=GRN_No&order=DESC&startrow=1&type=basic&search=lipase

[8] 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

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.

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 4). *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, once it has been verified that the NOAEL derived from existing toxicological studies is sufficiently high to provide adequate margin of exposure.

6.3.3 Safety Studies

Aspergillus niger lysophospholipase is an enzyme preparation produced from *T. reesei* that can be used as a processing aid in carbohydrate processing.

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 4) indicates that, regardless of the production organism strain, all enzyme preparations were found to have the following conclusions:

Antibiotic Resistance Marker Genes in Transgenic Plants (https://www.gpo.gov/fdsys/pkg/FR-1998-09-08/pdf/98-24072.pdf)

[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

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 4).

Based on strain lineage, the production strain most closely related to the AnLPL lysophospholipase production strain, is a *T. reesei* strain producing *T. reesei* trehalase. Toxicology studies with the trehalase from *T. reesei* have been conducted, and the data can be extrapolated to *T. reesei* lysophospholipase from *A. niger*. This approach is in line with the Safe Strain Lineage concept (Pariza and Johnson, 2001) endorsed by the Enzyme Technical Association (Sewalt *et al*., 2016). 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:

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.:	TRH-17004
Physical:	Fermentation liquid, brown
Enzyme:	Trehalase (CAS # 9025-52-9)
Enzyme activity:	405,309 U/g
pH:	4.21
Specific gravity:	1.09 g/ml
Total protein (TP):	270.74 mg/ml
TOS:	30.74 %

The studies include:

A. Repeated Dose 90-day Oral (Gavage) Toxicity Studay in CD Rats
B. *In vitro* Mammalian Chromosomal Aberration Assay in Human Peripheral Blood Lymphocytes (HPBL)
C. Bacterial Reverse Mutation Assay-Ames Assay

Summaries are included below.

A. Repeated Dose 90-day Oral toxicity in rats (Haskell Lab, 2018).

The objective of this study was to investigate the potential toxicity of *T. reesei* trehalase (Test article, H-32153) to induce systemic toxicity after repeated daily oral administration to Charles River CD rats of both sexes for 90 continuous days. This study was conducted in accordance with OECD guideline No. 408 (September 1998).

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. Under the conditions of this study, the no-observed-adverse-effect-level (NOAEL) was established at the high dose (1,000 mg TOS/kg BW).

B. *In vitro* Mammalian Chromosomal Aberration Assay in Human Peripheral Blood Lymphocytes (HPBL) (Haskell Lab, 2017).

The purpose of this study was to evaluate the potential of Trehalase 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. A preliminary toxicity test was performed to establish the dose range for testing in the cytogenetic test. This assay was conducted in accordance with OECD guideline No. 473 (2016). Under the conditions of the assay described in this test, Trehalase was concluded to be negative for the induction of structural and numerical chromosome aberrations in both the non-activated and S9-activated test systems. Trehalase was negative in the *In Vitro* Mammalian Chromosome Aberration Assay in HPBL.

C. Bacterial reverse mutation assay (Ames assay) (Haskell Lab, 2017).

The test article, Trehalase was tested in the Bacterial Reverse Mutation Assay using *Salmonella typhimurium* tester strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* tester strain

WP2 uvrA in the presence and absence of Aroclor-induced rat liver S9. This assay was conducted in accordance with OECD guideline No. 471 (1997). 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, Trehalase 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.

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 808 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 *Aspergillus niger* lysophospholipase is through oral ingestion, selection of this NOAEL is thus appropriate.

NOAEL: 1000 mg TOS/kg bw/day = 808 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.

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

$$\text{Margin of Safety} = \frac{1000 \text{ mg TOS/kg bw/day}}{0.087 \text{ mg TOS/kg bw/day}}$$

Margin of Safety = 11494

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, glucoamylase, alpha-glucosidase, transglucosidase, trehalase, 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 Nutrition & 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 based on repeated testing and evaluation using the Pariza and Johnson (2001) decision tree.

The exposure of the *A. niger* lysophospholipase produced by *T. reesei* as a food processing aid in carbohydrate processing is supported by toxicological data.

Based on a worst-case scenario that a person is consuming AnLPL lysophospholipase from the products of carbohydrate processing containing the lysophospholipase, the cumulative daily exposure of 0.087mg TOS/kg bw/day.

The margin of safety was calculated to be 11494 based on a NOAEL of 1000 mg TOS/kg bw/day based on the toxicological studies from *T. reesei* strain producing *T. reesei* trehalase. In the rare case of ingestion of the AnLPL lysophospholipase enzyme preparation, it is not expected to pose 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 AnLPL lysophospholipase as a processing aid in the carbohydrate processing including high fructose corn syrup to be used in food 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 Nutrition & Biosciences), and the decision tree analysis using generally recognized evaluation methodology (Pariza and Johnson, 2001; Sewalt *et al.*, 2016), the company has concluded that the *Aspergillus niger* lysophospholipase produced by *T. reesei* strain is safe and suitable for use as processing aid in the carbohydrate procesing. Collectively, the use of published information and evaluation methods provide a strong

common knowledge element, based upon which this AnLPL lysophospholipase can be considered Generally Recognized as Safe (GRAS) for its intended uses.

7. SUPPORTING DATA AND INFORMATION

7.1 LIST OF THE APPENDIXES

Appendix 1: The Amino Acid Sequence of the AnLPL

Appendix 2: The Manufacturing Process

Appendix 3: Certificate of Analysis (3 lots)

Appendix 4: *Trichoderma reesei* Strain Lineage and Summary of Safety Studies

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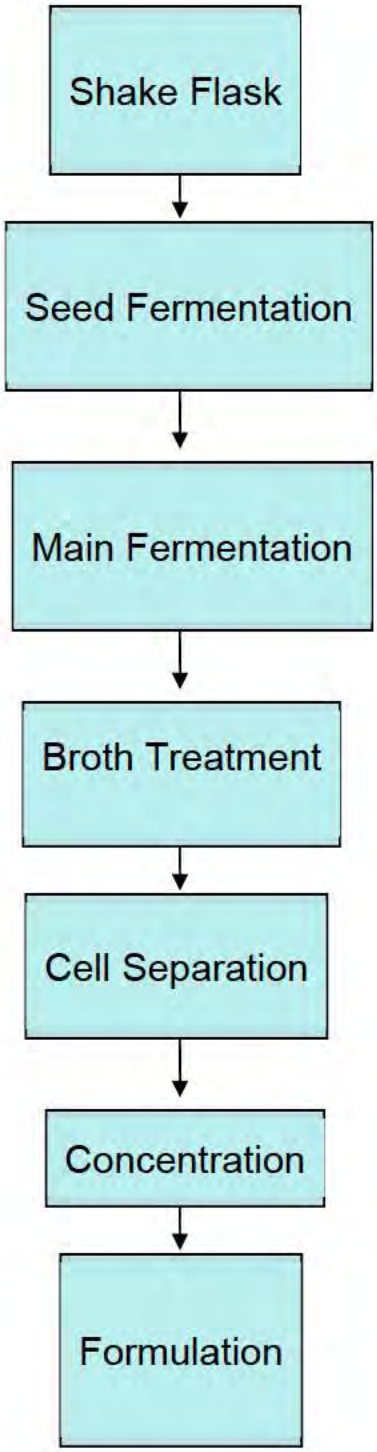
Appendix 1: The Amino Acid Sequence of the *Aspergillus niger* Lysophospholipase produced by *Trichoderma reesei*

ASLPVERAEAEVASVAADLIVRALPNAPDGYTPSNVTCPSTRPSIRDASGISTNETEWLKVRRNATLTPMKNLLSRLNLT
GFDTTSYINEHSSNISNIPNIAIAASGGGYRALTNGAGALKAFDSRSDNATNSGQLGGLLQAATYVSGLSGGSWLVGSM
FVNNFSSIGELQASEKVWRFDKSLLEGPNFDHIQIVSTVEYWKDITEEVDGKANAGFNTSFTDYWGRALSYQLVNASDD
KGGPDYTWSSIALMDDFKNGQYPMPIVVADGRNPGEIIVETNATVYEVNPWEFGSFDPSVYAFAPLQYLGSRFENGSI
PDNGTCVSGFDNAGFIMGSSSTLFNQFLLQINSTSIPTILKDAFTDILEDLGERNDDIAVYSPNPFSGYRDSSEDYATAKDL
DVVDGGEDGENIPLHPLIQPERAVDVIFAIDSSADTDYYWPNGTSLVATYERSLEPSIANGTAFPAVPDQNTFVNLGLNS
RPTFFGCDPKNISGTAPLVIYLPNSPYTYDSNFSTFKLTYSDEERDSVITNGWNVVTRGNGTVDDNFPSCVACAILQRSTY
RTNTSLPDICTTCFNDYCWNGTTNSTTPGAYEPSVLIATSGAIKSVLDYSVLALAMGVAAFML

Appendix 2: Manufacturing Process of the *Aspergillus niger* Lysophospholipase produced by *Trichoderma reesei*



Appendix 3: Certificate of Analysis (3 lots)

CERTIFICATE OF ANALYSIS

PRODUCT: **T-LPL UFC**
LOT NUMBER: **1663419842**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITY			
Lysophospholipase	U/g	Report value	427,455
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/ml	0 – 50000	<100
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
PHYSICAL PROPERTIES			
Specific gravity		Report	1.14
OTHER ASSAYS			
Lead	mg/kg	0 – 5	<5
Arsenic	mg/kg	0 - 3	<3
Cadmium	mg/kg	0 – 0.5	<0.5
Mercury	mg/kg	0 – 0.5	<0.5
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-Dec-2019
Date

Kelly A. Altman
QA/QC Department

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

CERTIFICATE OF ANALYSIS

PRODUCT: **T-LPL UFC**
LOT NUMBER: **GS20182463**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITY			
Lysophospholipase	U/g	Report value	1,720,990
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/ml	0 – 50000	<1
Coliforms	CFU/ml	0 - 30	<1
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
PHYSICAL PROPERTIES			
Specific gravity		Report	1.09
OTHER ASSAYS			
Lead	mg/kg	0 – 5	<5
Arsenic	mg/kg	0 - 3	<3
Cadmium	mg/kg	0 – 0.5	<0.5
Mercury	mg/kg	0 – 0.5	<0.5
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-Dec-2019
Date

Kelly A. Altman
QA/QC Department

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

CERTIFICATE OF ANALYSIS

PRODUCT: **T-LPL UFC**
LOT NUMBER: **GS20182464**

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITY			
Lysophospholipase	U/g	Report value	1,702,467
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/ml	0 – 50000	<1
Coliforms	CFU/ml	0 - 30	<1
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
PHYSICAL PROPERTIES			
Specific gravity		Report	1.09
OTHER ASSAYS			
Lead	mg/kg	0 – 5	<5
Arsenic	mg/kg	0 - 3	<3
Cadmium	mg/kg	0 – 0.5	<0.5
Mercury	mg/kg	0 – 0.5	<0.5
Mycotoxins		Negative by test	Negative

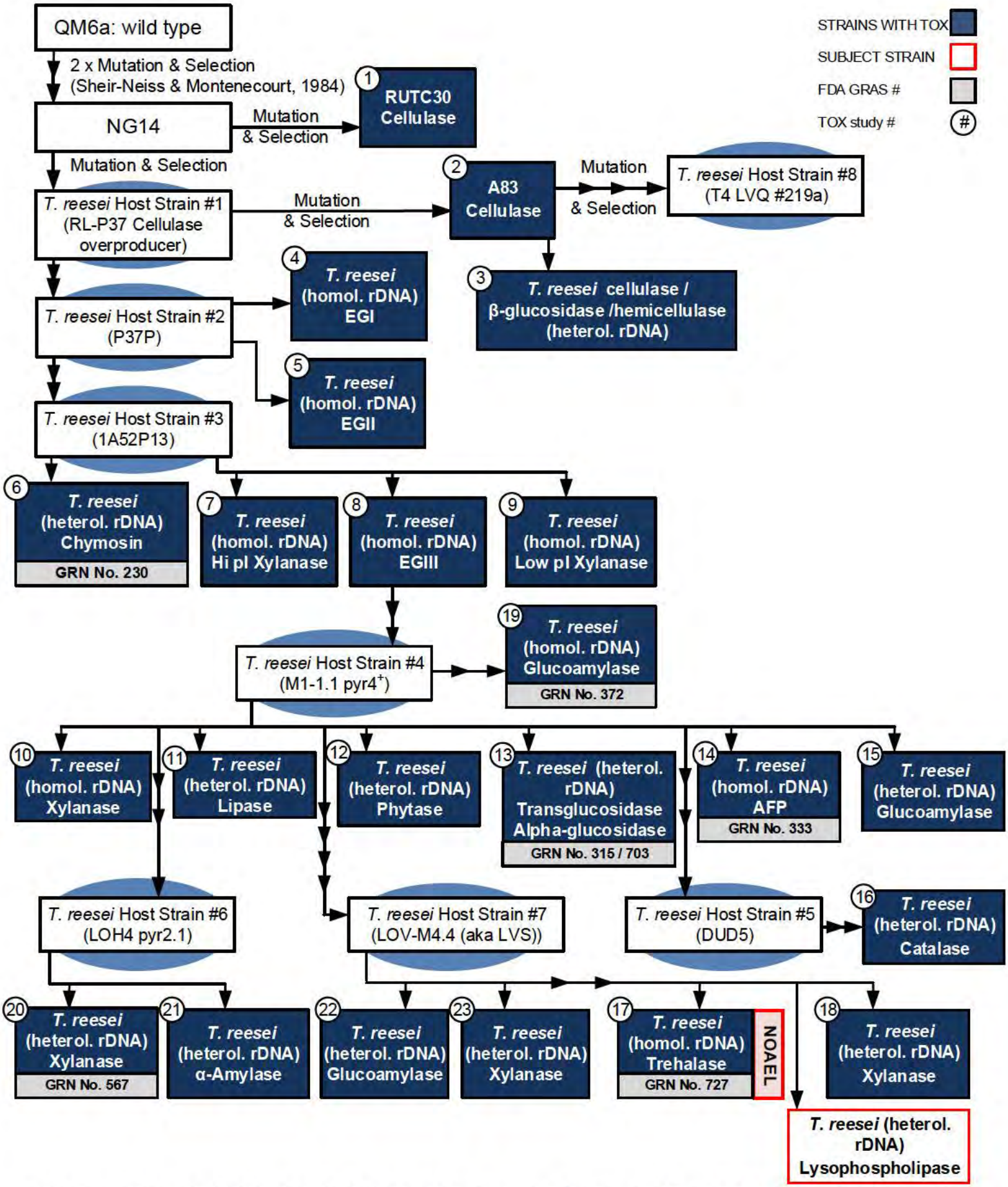
This product complies with the FAO/WHO and Food Chemicals Codex recommended specifications for food grade enzymes and contains permitted levels of stabilizers and preservatives.

16-Dec-2019
Date

Kelly A. Altman
QA/QC Department

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

Appendix 4: *Trichoderma 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 gray horizontal banners indicating the GRAS Notice number.The subject strain of this submission is the **lysophospholipase** producing strain highlighted in red.

The safety of the **lysophospholipase** enzyme is fully supported by repeated testing of enzymes produced by members of this Safe Strain Lineage. The dark blue coloured boxes indicate strains for which we conducted toxicology studies. The NOAEL for **trehalase** is used to calculate its safety margin in the intended uses.

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 21 enzyme preparations derived from the 21 recombinant production strains were assessed in several 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* (homologous 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* (homologous 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* (homologous 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* (homologous rDNA)	Endoglucanase III	28-Day subacute oral toxicity study, rats	No adverse effects
		Bacterial reverse mutation assay (Ames)	Not mutagenic
VII. *T. reesei* (homologous 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* (homologous 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* (homologous 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* (homologous 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)	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
XIX. *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
XX. *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
XXI. *T. reesei* (heterologous rDNA)	Xylanase (NGX)	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

XXII. *T. reesei*	Fungal Xylanase (FAX)	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
XXIII. *T. reesei* (heterologous rDNA)	Trehalase	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

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE (Subpart E of Part 170)

Form Approved: OMB No. 0910-0342; Expiration Date: 09/30/2019
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
000964	Aug 6, 2020
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration,5001 Campus Drive, College Park, MD 20740-3835.

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

☒ New ☐ Amendment to GRN No. _____ ☐ Supplement to GRN No. _____

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3 Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd):* _____

4 For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd):* _____

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position or Title
Vincent Sewalt	Senior Director, Product Stewardship & Regulatory
Organization *(if applicable)*	
Danisco US Inc. (operating as DuPont Nutrition & Biosciences)	
Mailing Address *(number and street)*	
925 Page Mill Road	

City	State or Province	Zip Code/Postal Code	Country
Palo Alto	California	94304	United States of America

Telephone Number	Fax Number	E-Mail Address
650-846-5861	650-845-6502	vincent.sewalt@dupont.com

1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position or Title
Annie Han	Senior Regulatory Affairs Specialist
Organization *(if applicable)*	
Danisco US Inc. (operating as DuPont Nutrition & Biosciences)	
Mailing Address *(number and street)*	
925 Page Mill Road	

City	State or Province	Zip Code/Postal Code	Country
Palo Alto	California	94304	United States of America

Telephone Number	Fax Number	E-Mail Address
650-846-4040	650-845-6502	annie.han@dupont.com

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

Lysophospholipase enzyme preparation from Trichoderma reesei expressing lysophospholipase gene from Aspergillus niger

2. Submission Format: *(Check appropriate box(es))*

☒ Electronic Submission Gateway ☐ Electronic files on physical media

☐ Paper

If applicable give number and type of physical media

_____

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in CFSAN's files? *(Check one)*

☐ Yes *(Proceed to Item 5)* ☒ No *(Proceed to Item 6)*

5. The submission incorporates information from a previous submission to FDA as indicated below *(Check all that apply)*

☐ a) GRAS Notice No. GRN _____

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☐ e) Other or Additional *(describe or enter information as above)* _____

6. Statutory basis for conclusions of GRAS status *(Check one)*

☒ Scientific procedures *(21 CFR 170.30(a) and (b))* ☐ Experience based on common use in food *(21 CFR 170.30(a) and (c))*

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? (see 21 CFR 170.225(c)(8))

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

SECTION D – INTENDED USE

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

The enzyme is lysophospholipase (IUBMB 3.1.1.5) which hydrolyzes 2-lysophosphatidylcholine to release glycerophosphocholine and carboxylate. This enzyme is intended to be used as processing aid in carbohydrate processing at 24.16 mg TOS/kg RM (raw material).

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☒ No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☐ No , you ask us to exclude trade secrets from the information FDA will send to FSIS.

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)

☒ PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).

☒ PART 3 of a GRAS notice: Dietary exposure (170.235).

☒ PART 4 of a GRAS notice: Self-limiting levels of use (170.240).

☒ PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).

☒ PART 6 of a GRAS notice: Narrative (170.250).

☒ PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☐ Yes ☒ No

Did you include this other information in the list of attachments?

☐ Yes ☐ No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Danisco US Inc.
(name of notifier)

has concluded that the intended use*(s)* of Lysophospholipase enzyme preparation from Trichoderma reesei expressing lysophospholipa
(name of notified substance)

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. Danisco US Inc. agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.
(name of notifier)

925 Page Mill Road, Palo Alto, CA 94304, USA
(address of notifier or other location)

The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance.The notifying party certifies that the information provided herein is accurate and complete to the best or his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

3. Signature of Responsible Official, Agent, or Attorney	**Printed Name and Title**	**Date (mm/dd/yyyy)**
annie.han@dupont.com Digitally signed by annie.han@dupont.com DN: cn=annie.han@dupont.com Date: 2019.12.17 14:16:55 -08'00'	Annie Han, Senior Regulatory Affairs Specialist	12/17/2019

SECTION G – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Form3667_GRASNotive_LysophosponlipaseFromTrichodermaReesei_2019-12-17.pdf	Administrative
	GRASNotice_LysophospholipaseFromTrichodermaReesei_2019-12-17.pdf	Submission

OMB Statement: Public reporting burden for this collection of information is estimated to average 170 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services, Food and Drug Administration, Office of Chief Information Officer, **PRAStaff@fda.hhs.gov**. (Please do NOT return the form to this address). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.