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
000808	8/17/2018
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
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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 Industrial 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 Industrial 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

Lipase enzyme preparation from Trichoderma reesei expressing lipase gene from Aspergillus tubingensis

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 lipase (IUBMB 3.1.1.3) which hydrolyzes ester bonds primarily in the 1- and 3- position of fatty acids in triglycerides with release of fatty acids and glycerol. The enzyme also has activity towards sn-1 ester bonds in other lipid components including diacyl-phospholipids and diacyl-galactolipids. This enzyme is intended to be used as processing aid in baking at 21.2 mg TOS/kg RM (raw material), in the brewing process and manufacture of cereal beverage at 52.2 mg TOS/kg RM, in pasta production at 2.03 mg TOS/kg RM, and in potable alcohol production at 3.6 mg TOS/kg RM.

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 Lipase enzyme preparation from Trichoderma reesei expressing lipase gene from Aspergillus t
(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)
[redacted]	Annie Han, Senior Regulatory Affairs Specialist	08/17/2018

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_LipaseFromTrichodermaReesei_2018-08-17.pdf	Administrative
	GRASNotice_LipaseFromTrichodermaReesei_2018-08-17.pdf	Submission

A Lipase Enzyme

Preparation Derived from

Trichoderma reesei

Expressing the Lipase Gene

From

Aspergillus tubingensis

Is Generally Recognized As Safe

For Use in Food Processing

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

August 17, 2018

TABLE OF CONTENT

1. GENERAL INTRODUCTION, STATEMENT AND CERTIFICATION

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

The lipase enzyme is intended for use to hydrolyze triacyglycerol. It hydrolyzes ester bonds primarily in the 1- and 3- position of fatty acids in triglycerides with release of fatty acids and glycerol. The enzyme also has activity towards sn-1 ester bonds in other lipid components including diacyl-phospholipids and diacyl-galactolipids. The lipase enzyme will be used in baking and brewing process, in the manufacture of cereal beverage, in pasta production, and in potable alcohol production. In these applications, the lipase 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 triacylglycerol acylhydrolase. The IUBMB nomenclature is triacylglycerol lipase. Other names used are triacylglycerol acyl hydrolase, triacylglycerol ester hydrolase, etc., as described in Section 2.2.1 of this submission. For consistency, this enzyme will be presented by the name “Lipase 3” throughout the dossier.

The enzyme hydrolyzes triacyglycerol with release of diacylglycerol and carboxylate.

The EC number of the enzyme is 3.1.1.3, and the CAS number is 9001-62-1.

The information provided in the following parts is the basis of our determination of GRAS status of this Lipase 3 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].

The safety of the production organism is considered to be prime consideration in assessing the safety of an enzyme preparation intended for food use (Pariza & Johnson, 2001; Pariza & Foster,

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

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 Industrial Biosciences)
925 Page Mill Road
Palo Alto, CA 94304

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

The Lipase 3 enzyme preparation is produced in a *Trichoderma reesei* strain expressing the gene encoding the lipase from *Aspergillus tubingensis.*

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

The Lipase 3 is intended to be used as a processing aid in baking at 21.2 mg TOS/kg RM (raw material), in the brewing process and manufacture of cereal beverage at 52.2 mg TOS/kg RM, in pasta production at 2.03 mg TOS/kg RM, and in potable alcohol production at 3.6 mg TOS/kg starch.

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. §170.3225, Danisco US Inc. has determined that its Lipase 3 enzyme preparation from a genetically engineered strain of *T. reesei* expressing the lipase enzyme from *A. tubingensis* 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.

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.

August 10, 2018

Vincent Sewalt
Senior Director, Product Stewardship & Regulatory
Danisco US Inc. (Operating by DuPont Industrial Biosciences)
925 Page Mill Road
Palo Alto, CA 94304
Work: 650-846-5861
Mobile: 650-799-0871
Email: vincent.sewalt@dupont.com

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 Lipase 3 gene from *A. tubingensis*.

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. tubingensis* Lipase 3 gene regulated under the expression signals of the endogenous *Trichoderma reesei cbh1* gene, and multiple copies of the expression cassette were integrated into the recipient chromosome using the *Aspergillus nidulans* acetamidase (*amdS*) gene as a 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 Lipase 3 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 *A. tubingensis* Lipase 3 into the *T. reesei* host.

The expression cassette comprised:

- The native *T. reesei* cellobiohydrolase (*cbhI*) promoter, which was used to drive expression of the *A. tubingenesis* Lipase 3 gene,
- The native *A. tubingensis* Lipase 3 gene encoding its native (unmodified) amino acid sequence,
- the native *T. reesei* cellobiohydrolase (*cbhI*) terminator,
- And the *A. nidulans* acetamidase gene (*amdS)* 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 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 Lipase 3 gene in the production strain proved to be 100% stable after industrial scale fermentation as judged by lipase 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)[1] is satisfactorily addressed.

[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:	Lipase
IUB Nomenclature:	Triacylglycerol lipase
IUB Number:	3.1.1.3
CAS Number:	9001-62-1
Reaction catalyzed:	hydrolyze ester bonds primarily in the 1- and 3- position of fatty acids in triglycerides with release of fatty acids and glycerol

2.2.2 Amino Acid Sequence

The amino acid sequence of the *A. turbingensis* Lipase 3 is known and included in Appendix 1.

2.3 MANUFACTURING PROCESS

This section describes the manufacturing process for this Lipase 3 enzyme which follows standard industry practice (Kroschwits, 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 Lipase 3 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, 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) 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 and flocculants. Therefore, the maximum use level of the antifoams in the production process is <1.0%, and cationic polymer flocculants < 1.

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. The final dry products for the bakery applications can be spray-dried on potato- or wheat starch but since bakery products are produced with similar allergen group (*e.g.*, wheat), no additional allergens are introduced into the final food. Therefore, the final enzyme preparation does not introduce any new major food allergens from the fermentation medium into the final food. No other major allergen substances are used in the fermentation, recovery processes, or formulation of this product.

2.3.2 Fermentation Process

The Lipase 3 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 (UFC) is stabilized by final formulation to contain 25-30% enzyme, 0.2% sodium benzoate, and 0.2% potassium sorbate at pH 4.3-5.0. The remaining portion of the UFC formulation is water.

The final Lipase 3 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.

Lipase 3	25-30%
Potassium sorbate	0.2%
Sodium benzoate	0.2%

The preparation includes TOS (total organic solids resulting from the fermentation), which is approximately 26% of the liquid concentrate.

Various commercial formulations exist, with a range of enzyme activities. The following is a representative composition for spray-dried commercialized product:

Lipase 3	50-80%
Potato starch	15-40%
Water	5-7.5%
Potassium sorbate	0-0.55%
Sodium benzoate	0-0.55%

2.4.2 Specifications

As mentioned, Lipase 3 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

Lipase catalyzes the hydrolysis of ester bonds in triglycerides primarily in 1 and 3 positions of fatty acids in triglycerides with release of fatty acids and glycerol.

2.5.2 Use Levels

The Lipase 3 preparation is intended for use in baking and brewing process, in the manufacture of cereal beverage, in pasta production, and in potable alcohol production.

The table below shows the recommended use levels for each application where the Lipase 3 may be used.

Application	**Recommended Use Level (mg TOS/kg Raw Material)**
Baking	21.2
Brewing	52.2
Cereal beverage	52.2
Pasta	2.03
Potable alcohol	3.6

2.5.3 Enzyme Residues in the Final Foods

The Lipase 3 enzyme will be deactivated or removed during the subsequent production and refining processes for all applications. In the rare case that inactive lipase 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

Lipase 3 will be used as a processing aid in baking and brewing, in the manufacture of cereal beverage, in pasta production, and in potable alcohol production.

While we expect the Lipase 3 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 Lipase 3 via baking, brewing, cereal beverage, pasta, and potable alcohol 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 levels of the enzyme Lipase 3 are 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 Lipase 3 in the applications can be calculated/summarized as in the table below:

	Application	**Raw Material (RM)**	**Maximal recommended use level (mg TOS/kg RM)**	**Example Final food (FF)**	**Rate RM/FF**	**Maximal level in FF (mg TOS/kg food)**
Liquid Food	Potable Alcohol	Cereal	3.6	Potable alcohol	1/0.35 =2.86	10.29
	Brewing processes	Wort	52.2	Beer	0.17	8.87
	Cereal beverage	Wort	52.2	Cereal beverage	0.17	8.87
Solid Food	Baking	Flour	21.2	Bread Bun Cakes etc.	0.71	15.05
	Pasta	Flour	2.03	Pasta Noodle	1	2.03

However, for the purpose of selecting an overall maximum exposure via the consumption of liquids, the worst-case TOS concentration in brewing or cereal processing (8.87 mg TOS/L) is appropriate for liquid food, because in distilled spirits, the actual TOS concentration will be minimal compared to the maximum theoretical TOS concentration, as the enzyme protein and other organic solids will be removed in the distillation step, and consumption of distilled spirits is

self-limiting resulting in significantly lower exposure. For the purpose of selecting an overall maximum exposure via the consumption of solid food, the worst-case TOS concentration in baking (15.05 mg TOS/kg) is appropriate.

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 Lipase 3 from brewing or cereal beverage 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 Lipase 3. 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 brewing or cereal beverage (worst case)	8.87	mg TOS/L beverage
TMDI beverages	0.222	mg TOS/kg bw/day

Estimation of the TMDI for Solid Foods

The maximum dosage used in baking application is used for representation of worst-case scenario for solid food.

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	15.05	mg TOS/kg final food
TMDI solid food	0.188	mg TOS/kg bw/day

The Theoretical Maximum Daily Intake (TMDI)- total

TMDI $_{\text{beverages}}$	0.222	mg TOS/kg bw/day
TMDI $_{\text{Solid food}}$	0.188	mg TOS/kg bw/day
TMDI total	0.410	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 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, 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 Lipase 3 enzyme preparation, the subject of this submission, is *T. reesei* strain Morph Lip3, which was produced from strain RL-P37 using recombinant DNA methods. The purpose of this genetic modification is to express the lipase from *A. tubingensis* 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

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

all over the world for industrial cellulase production today are derived from QM6a. Danisco US, Inc. (operating as DuPont Industrial 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, and GRN 727).[1]

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 Lipase 3. The strain is non-pathogenic and non-toxigenic.

6.1.2 Safety of the donor source

The donor strain used as a source for the Lipase 3 gene was *Aspergillus tubingensis* (DuPont IB strain 1M341). *Aspergillus tubingensis* is also called *Aspergillus niger var. tubingensis*. It is one of the species in the *Aspergillus* section Nigri (the black aspergilli). In the *Aspergillus niger aggregate*, although speciation at molecular level has been proposed, no morphological differences can be observed and species identification will therefore remain problematic.

The species *Aspergillus niger var. tubingensis* is a deuteromycetes with a full taxonomic lineage as:

> *cellular organisms; Eukaryota; Opisthokonta; Fungi; Dikarya; Ascomycota; saccharomyceta; Pezizomycotina; leotiomyceta; Eurotiomycetes, Eurotiomycetidae, Eurotiales, Aspergillaceae, Aspergillus, Aspergillus niger; Aspergillus niger var. Tubingensis*

Aspergillus niger var. tubingensis is a fungus of the genus *Aspergillus*. Black-spored Aspergillus section Nigri species has been identified for production of the mycotoxins ochratoxin A (OTA) and fumonisin B2 (FB2) which are toxic for human and animals. Ochratoxins and fumonisins are a small group of chemically related toxic fungal metabolites (mycotoxins).

A review of the abstracts revealed grapes (for wine and raisins) are the most commonly *Aspergillus* contaminated crop (Medina *et al.*, 2005), and ochratoxin A is the most reported mycotoxin associated with *Aspergillus* species (*Aspergillus niger* being identified as the main source of

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

Ochratoxin A). *Aspergillus* species has also been associated with myctoxin production in maize (Logrieco *et al*., 2014) and citrus fruits (Kanetis *et al*., 2015). *Aspergillus tubingensis* was identified in maize but not reported for mycotoxin production (Logrieco *et al*., 2014).

Storari *et al.* (2012) has assessed six *Aspergillus tubingensis* strain from International culture collections for ochratoxin A (OTA) production. OTA was not detected in any of the tested samples. The non-toxigenic nature of *Aspergillus tubingensis* is further supported by several reports (Frisvad *et al.*, 2011; Accensi *et al*., 2001; Nielsen *et al*., 2009 and others).

An article by Bathoorn *et al*. (2013) reported *Aspergillus tubingensis* infections in immunocompromised patients.

Aspergillus nidulans acetamidase (*amdS*) gene was used as a selectable marker, to enable growth on acetamide medium. Only the *amdS* gene in isolated form was used. The gene was first described by Hynes *et al*. (1983). The strain was not described further than "a strain of genotype biA1" but it is certainly a derivative of the original *Aspergillus nidulans* isolate (Glasgow wild-type) deposited as strain A4 at the Fungal Genetics Stock Center, Kansas City, USA. Meanwhile, the description of the gene in GenBank (Accession number M16371) mentions the Glasgow wild-type *Aspergillus nidulans* strain as the source. Sequencing and PCR experiments verified that the gene Danisco US Inc. used is the same as published by Corrick *et al*. (1987).

The donor strain was not directly used as a source of introduced DNA, but only DNA fragments encoding the known enzyme activity were obtained from the donor organisms' chromosomal DNA by PCR synthesis and used in the final production organism.

6.2 SAFETY OF THE MANUFACTURING PROCESS

The manufacturing process to produce Lipase 3 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).

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 dry products for the bakery applications can be spray-dried on potato- or wheat starch but since bakery products are produced with similar allergen group (*e.g.*, wheat), no additional allergens are introduced into the final food. 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.

6.3 SAFETY OF LIPASE 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 Lipase 3 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 lipase 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 tubingensis* Lipase 3 (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] March 23, 2018 V18B, which contains 2089 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 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, *A. tubingensis* Lipase 3is 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 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.

In addition to the allergenicity assessment described above, the safety of this Lipase 3 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, lipase has been used for years in food processing. The *Aspergillus tubingensis* Lipase 3 is relatively new as an isolate in food processing. However,

[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.

it has high homology to other lipases used in food - *e.g.*, 98% identity with *Aspergillus niger* lipase (GRN 111, GRN 296), 99% identity with *Aspergillus kawachii* lipase, and 56% with *Aspergillus oryzae* lipase (GRN 113), and its protein sequence is not similar to known sequences of food allergens and toxins. Go to 3c.

3c. Is the test article free of transferable antibiotic resistance gene DNA[1]? 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.

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure[2]? 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.

[1] 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 (https://www.gpo.gov/fdsys/pkg/FR-1998-09-08/pdf/98-24072.pdf)

[2] 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

6.3.3 Safety Studies

Aspergillus tubingensis Lipase 3 is an enzyme preparation produced from *T. reesei* that can be used as a processing aid in in baking and brewing process, in the manufacture of cereal beverage, in the pasta production, and in the potable alcohol production.

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:

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).

In addition to the decision tree analysis and the availability of multiple toxicology studies for the safe strain lineage, different endpoints of toxicity of this lipase were investigated as part of our safety program to satisfy international and external requirements globally. This battery of tests included:

1) Acute oral toxicity study in rats,
2) Bacterial reverse mutation assay,
3) Genotoxicity studies (Ames assay and chromosomal aberration tes), and
4) 90-day oral toxicity studay in rats

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.:	
Physical:	Fermentation liquid, brown
Enzyme:	Lipase (CAS # 9001-62-1)
Enzyme activity:	108385 LIPU/g

pH:	6.17
Specific gravity:	1.055 g/ml
Total protein (TP):	178.40 mg/ml
TOS:	13.68 %

A. **Acute oral Toxicity Study in Rats – up and down Procedure. MB Research Laboratories (Pennsylvania), Study No. 1010-2, August 5, 2009.**

a. Procedure:

The objective of this study was to assess the acute toxicity of the Lipase 3 when administered as a single oral dose followed by a 14-day period of observation. The information is used for both hazard assessment and ranking purposes. The study was initiated with a single female Wistar rat at 2000 mg/kg. Since this animal survived, the study was followed with four additional female rats dosed at 2000 mg/kg bw.

This study was conducted according to EPA Health Effects Test Guidelines, OPPTS 870.1100 (December 2002) and the OECD Guideline No. 425 (updated March 2006) and in compliance with Good Laboratory Practices regulations of the EPA 40 C.F.R. § § 160 and 792, FDA 21 C.F.R. §58, and as specified in Principles on Good Laboratory Practices published by OECD, 1997.

b. Results

No mortality was recorded in this study at 2000 mg/kg bw. There were no abnormal physical signs noted during the observation period. There were no abnormal findings at necropsy.

c. Evaluation

Under the conditions of this study, the oral LD_{50} was $\geq$2000 mg total protein/kg bw (corresponding to 1540 mg TOS/kg bw). Based on a LD_{50} > 2000 mg/kg, Lipase 3 is classified to category 5 [unclassified practically nontoxic] according to the Globally Harmonized System of Classification and Labeling of Chemicals (GHS), 2007.

B. **Bacterial Reverse Mutation Assay – Ames assay. Harlan Laboratories (Switzerland), Study No. 1261601, July 3, 2009.**

a. Procedure

The objective of this assay was to assess the potential of Lipase 3 to induce point mutations (frame-shift and base-pair) in four strains of *Salmonella typhimurium* TA 98, TA 100, TA 1535 and TA 1537 and *Escherischia coli* strain WP2 uvrA. The test material was tested both in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). A pre-experiment test was performed with strains TA 98, TA 100, TA 1535, TA 1537 and WP2 uvrA using 8 concentrations ranging from 3 to 5,000 µg/plate. All dose levels were expressed in terms of total protein. The highest dose level tested (5,000 µg/plate) is the maximum required by the OECD guideline. Subsequently, a main test was performed with all 5 strains in both the presence and absence of S-9 mix. Triplicate plates were used at each test point. Eight dose levels of lipase were used in the main test and ranged from 3 to 5,000 µg/plate. The positive controls used for assays without S-9 mix were sodium azide, 4-nitro-o-phenylene-diamine and methyl methane sulfonate. The positive control for assays with S-9 mix was 2-aminoanthracene. Negative control plates were treated by the addition of sterile deionized water.

This assay was conducted in accordance with OECD guideline No. 471 (July 21, 1997), EPA OPPTS 870.5100 (August 1998) and complied with OECD Principles on GLP (as revised in 1997) and all subsequent OECD consensus documents.

b. Results

In the pre-experiment assay, this Lipase 3 was not toxic to the test bacteria up to and including the highest dose level (5,000 µg/plate) in both the absence and presence of S-9 mix. Therefore, 5000 µg/plate was selected as the highest dose level for the main test.

In the main test, eight dose levels (3; 10; 33; 100; 333; 1,000; 2,500 and 5,000 µg/plate) were tested. The plates incubated with the test material showed normal background growth up to 5,000 µg/plate with and without metabolic activation. No biologically significant increases in the number of revertant colonies were observed at any dose level of the test item. There was also no tendency of higher mutation rates with increasing concentrations of the test material. Statistical increases in the number of revertant colonies were noted with the positive controls in both the presence and absence of metabolic activation substantiating the sensitivity of the treat and plate assay and the efficacy of the metabolic activation mixture.

c. Evaluation

Under the conditions of this assay, Lipase 3 has not shown any evidence of mutagenic activity in the Ames assay. Lipase did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

C. ***In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes. Harlan Laboratories (Switzerland), Study No. 1261602, July 20, 2009.**

a. Procedure

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

Lipase 3 concentrate was mixed with cultures of human peripheral lymphocytes both in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver; S-9 mix). This assay consisted of a preliminary toxicity (dose range finding) assay and one main test. Ten concentrations of Lipase were used in the preliminary assay and at least 3 dose levels were then selected for analysis of chromosome aberration with the highest dose level clearly inducing a toxic effect (50% reduction in mitotic index). Cytotoxicity is characterized by the percentage of mitotic suppression in comparison to the controls by counting 1,000 cells per culture in duplicate. In the absence of cytotoxicity, the highest dose selected would be 5,000 µg/ml, as recommended by the OECD guideline.

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

i) Cytotoxicity was evaluated using the mitotic index (number of cells in mitosis/1,000 cells examined). From these results, a dose level causing a decrease in mitotic index of 50% was selected as the highest dose in the main assays.

ii) Metaphase analysis (*i.e.*, evaluation of chromosomal aberration) was conducted on at least 100 metaphases per culture dose level.
iii) Ethylmethane sulfonate and cyclophosphamide were used as positive controls for cultures without S-9 mix and cultures with S-9 mix, respectively.

This assay was conducted in accordance with OECD guideline No. 473 (*In vitro* Mammalian chromosome aberration test; February 1998) and complied with Commission Regulation (EC) No. 440/2008 B.10: "Mutagenicity – In Vitro Mammalian Chromosome Aberration Test" dated May 30, 2008. The study was performed in compliance with the Chemicals Act of the Federal Republic of Germany (July 25, 1994; revised June 27, 2002) and the OECD Principles of Good Laboratory Practice (1997).

b. Results

Preliminary assay (Experiment I): Ten dose levels ranging from 32.5 to 5,000 µg/ml were used. Exposure period was 4 hours for both cultures with and without S-9 mix. No clear cytotoxicity was observed up to the highest concentration tested 5,000 µg/ml. No visible precipitation of the test material in the culture medium was observed. No biologically relevant increases in cells with chromosomal aberrations were noted in three highest dose levels selected for analysis (1,632.7; 2,857.1 and 5,000 µg/ml). Since the cultures fulfilled the requirements for cytogenetic evaluation, this preliminary assay was designated as Experiment I and the results were analyzed for statistical significance.

Main assay (Experiment II: Exposure period was 4 hours for cultures with S-9 mix and 22 hours for cultures without S-9 mix. Ten dose levels ranging from 32.5 to 5,000 µg/ml were used. The chromosomes were prepared 22 hours after the start of treatment with the test material.

No visible precipitation of the test material in the culture medium was observed. In both the presence of S-9 mix (4-hour cultures) and absence of S-9 mix (22-hour cultures), no clear cytotoxicity was observed up to the highest concentration tested 5,000 µg/ml. No biologically relevant increases in cells with chromosomal aberrations were noted in three highest dose levels selected for analysis (1,632.7; 2,857.1 and 5,000 µg/ml).

In both experiments I and II, no increase in polyploidy metaphases was noticed.

In both experiments, significant increases in aberrant metaphases were demonstrated with the positive controls demonstrating the sensitivity of the tests and the efficacy of the S-9 mix.

c. Evaluation

Under the conditions of this test, the Lipase 3 did not induce chromosomal aberrations (both structural and numerical) in this *in vitro* cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation up to the highest concentration (5,000 µg/ml) recommended by guidelines.

D. **A 90-days Oral Toxicity (Gavage) Study in Wistar Rats. Harlan Laboratories (Switzerland), Study No. C64433, April 09, 2010.**

a. Procedure

The objective of this study was to investigate the potential of the lipase to induce systemic toxicity after repeated daily oral administration (gavage) to SPF-bred Wistar rats of both sexes. Dose levels were 0 (0.9% saline), 53.5, 80.3 and 160.6 mg total protein/kg bw/day (corresponding to, respectively, 0, 41.02, 61.57 and 123.15 mg TOS/kg bw/day or 0; 32,390; 48,610 and 97,225 LIPU/kg bw/day). Each group consisted of 10 animals/sex. Animals of the same sex were housed in groups of five in Makrolon-type 4 cages with wire mesh tops and softwood bedding and had access to water (via bottle) and feed *ad libitum*. For environmental enrichment, the animals were provided a supply of Aspen Wood Wool at each change of bedding. All groups were housed under controlled temperature, humidity, and lightning conditions.

All animals were observed daily for mortality and signs of morbidity. Body weight and feed consumption were recorded weekly. Ophthalmologic examination was performed on all animals prior to study initiation and at study termination. Urinalysis, clinical chemistry and hematology were conducted at week 13. A functional observation battery consisting of detailed clinical observation, reactivity to handling and stimuli and motor activity examination was conducted during week 13 for all groups. All animals were sacrificed at the end of the treatment period. After a thorough macroscopic examination, selected organs were removed, weighed and processed for future histopathologic examination. Microscopic examination was initially conducted on selected organs from control and high dose animals.

This study was conducted in accordance with OECD guideline No. 408 (September 1998) and Directive 96/54/EC, B.26. “Subchronic Oral Toxicity”, 30 September 1996 and in compliance with the Swiss Ordinance relating to Good Laboratory Practice (May 18, 2005) and the OECD Principles of Good Laboratory Practice (1997).

b. Results

One control female (# 49) was sacrificed for humane reasons on day 57 of treatment.

There was no mortality in the low dose group (53.5 mg total protein/kg bw/day). In the mid dose group (80.3 mg total protein/kg bw/day), one male was found dead on day 46 due to gavage error (presence of test material in the lungs) and two females were found dead on days 31 and 45. The cause of death of the two mid-dose females was not determinable but was not considered treatment-related in the absence of mortality noted in mid-dose males and high-dose males and females. In the high dose group (160.6 mg total protein/kg bw/day), gavage error resulted in the death of one female (presence of test material in lungs; dark red discoloration of lungs).

There were no treatment-related statistical differences between the control and treated groups with respect to clinical observation, functional observation, body weight gains, feed consumption, hematology, clinical biochemistry, and urinalysis. Significantly higher mean hind-limb strength values were noted at all dose levels. However, these differences were considered to be incidental in the absence of similar findings in fore-limb grip strength. Increased locomotor activity was noted in mid and high dose males but the differences were not dose related. The mean absolute neutrophil count and plasma sodium were significantly elevated in high dose males when compared to concurrent control values. However, these differences were not considered as treatment-related since they were within the historical control data values for this species and strain collected at the testing laboratory. Higher plasma glucose was found in high dose females but the values were still within the historical control data range. At necropsy, a small number of statistically significant differences to the control values were noted in the mean absolute and/or relative organ weights. However, in the absence of accompanying histopathologic and/or functional changes and clear dose response relationship, these variations are considered as incidental. All microscopic lesions were within the normal background range of lesions found in laboratory animals of this strain and age.

c. Evaluation and Conclusion

Daily administration of the Lipase 3 for 91/92 days by oral gavage to Wistar rats at doses of 0 (0.9% saline), 53.5, 80.3 and 160.6 mg total protein/kg bw/day (corresponding to, respectively, 0, 41.02, 61.57 and 123.15 mg TOS/kg bw/day or 0; 32,390; 48,610 and 97,225 LIPU/kg bw/day) resulted in no treatment-related deaths, clinical observations, feed consumption, body weight changes, hematology, clinical chemistry, urinalysis, organ weights, functional observation, grip strength and locomotor activities. No macroscopic or microscopic changes could be attributed to treatment.

Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established at the highest dose tested, 160.6 mg total protein/kg bw/day corresponding to 123.15 mg TOS/kg bw/day or 97,225 LIPU/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 123.15 mg Total Organic Solids (TOS) /kg bw/day equivalent to 160.6 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 tubingensis* Lipase 3 is through oral ingestion, selection of this NOAEL is thus appropriate.

NOAEL: 123.15 mg TOS/kg bw/day = 160.6 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.

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

$$\text{Margin of Safety} = \frac{123.15 \text{ mg TOS/kg bw/day}}{0.410 \text{ mg TOS/kg bw/day}}$$

Margin of Safety = 300

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 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 Lipase 3 from *T. reesei* as a food processing aid in baking and brewing process, in the manufacture of cereal beverage, in pasta production, and in potable alcohol production is assessed based on a battery of toxicology studies.

Although the enzyme is produced by a strain that belongs to a safe strain lineage, toxicological studies for the subject enzyme are available. Genotoxicity assays were conducted with this Lipase 3 and under the conditions of these assays *A. tubingensis* Lipase 3 is not classified as a mutagen, a clastogen, or an aneugen. The systemic toxicity of *A. tubingensis* Lipase 3 was investigated in an oral study (90-day) and daily administration of *A. tubingensis* Lipase 3 for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at 123.15 mg TOS/kg bw/day.

Based on a worst-case scenario that a person is consuming Lipase 3 from the products of baking, brewing, cereal beverage, pasta, and potable alcohol containing the lipase, the cumulative daily exposure of 0.410mg TOS/kg bw/day.

Based on a margin of safety (300) greater than 100 even in the worst-case, the proposed uses of Lipase 3 in baking and brewing process, in the manufacture of cereal beverage, in the pasta production, and in the potable alcohol production 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 the Lipase 3 from *T. reesei* strain is safe and suitable for use as processing aid in baking and brewing process, in the manufacture of cereal beverage, in pasta production, and in potable alcohol production. Collectively, the use of published information and evaluation methods provide a strong common knowledge element, based upon which this lipase 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 5).

7. SUPPORTING DATA AND INFORMATION

7.1 LIST OF THE APPENDIXES

Appendix 1: The Amino Acid Sequence of the Lipase 3

Appendix 2: The Manufacturing Process

Appendix 3: Certificate of Analysis (3 lots)

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

Appendix 5: External Expert Opinion Letter from Dr. Michael Pariza

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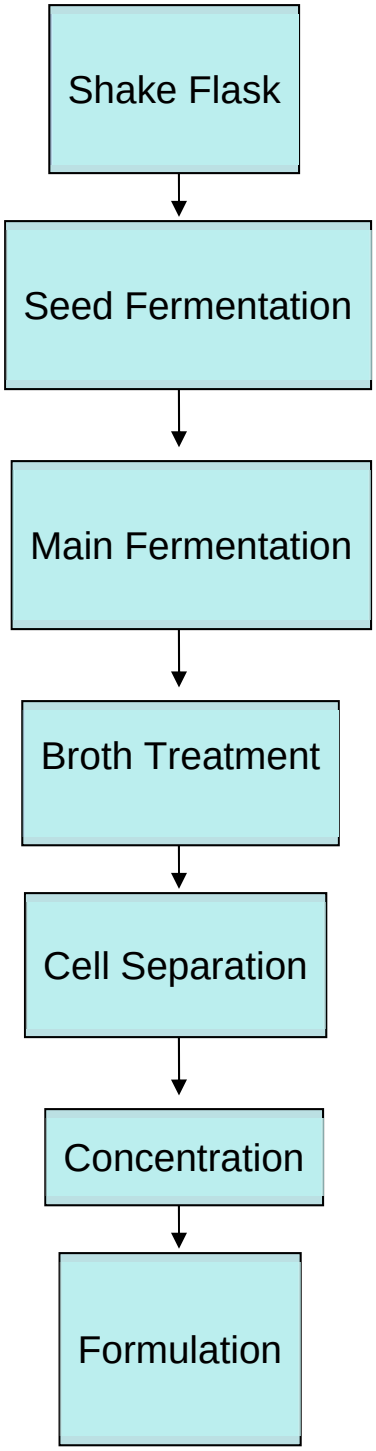
Appendix 1: Amino Acid Sequence of Lipase 3

APAPLAVRSVSTSTLDELQLFAQWSAAAYCSNNIDSKDSNLTCTANACPSVEEASTTMLLEFDLTNDFGGTAGFLAA
DNTNKRLVVAFRGSSTIENWIANLDFILEDNDDLCTGCKVHTGFWKAWESAADELTSKIKSAMSTYSGYTLYFTGHS
LGGALATLGATVLRNDGYSVELYTYGCPRIGNYALAEHITSQGSGANFRVTHLNDIVPRVPPMDFGFSQPSPEYWIT
SGNGASVTASDIEVIEGINSTAGNAGEATVSVVAHLWYFFAISECLL

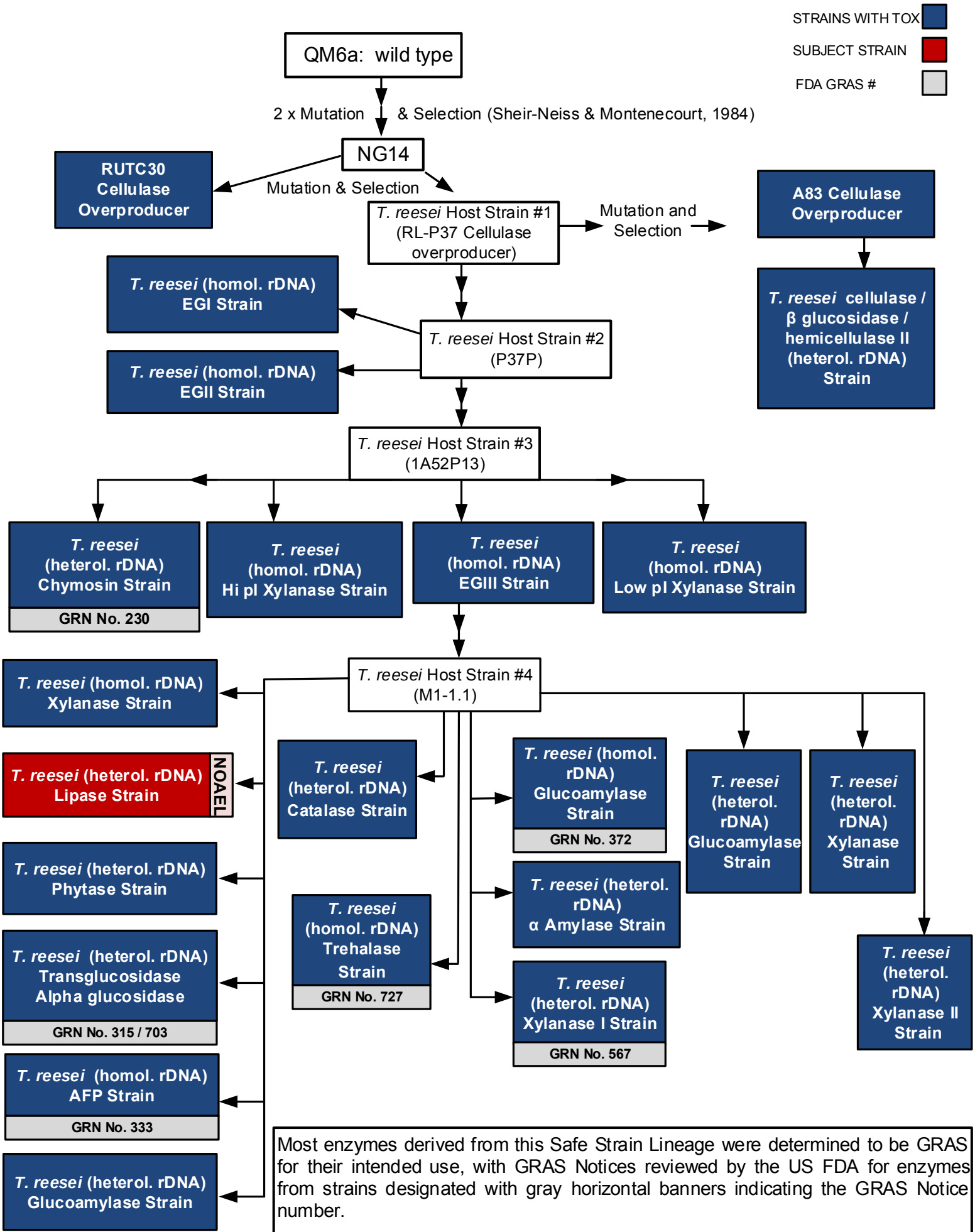
Appendix 2: Manufacturing Process of Lipase 3



Appendix 3 – CERTIFICATE OF ANALYSIS of Lipase 3 (3 lots)

Property	Lot number [redacted]	Lot number [redacted]	Lot number [redacted]
ENZYME ACTIVITIES			
Lipase	120000 LIPU/g	124000 LIPU/g	107901 LIPU/g
MICROBIAL ANALYSIS			
Total Viable Count	<1 CFU/ml	<1 CFU/ml	3 CFU/ml
Total Coliforms	<1 CFU/ml	<1 CFU/ml	<1 CFU/ml
E. coli	NEG/25 ml	NEG/25 ml	NEG/25 ml
Salmonella	NEG/25 ml	NEG/25 ml	NEG/25 ml
Production strain	NEG/ml	NEG/ml	NEG/ml
Antimicrobial Activity	NEG/ml	NEG/ml	NEG/ml
PHYSICAL PROPERTIES			
pH	4.35	4.22	4.5
OTHER ASSAYS			
Heavy Metals, as Pb	<30 mg/kg	<30 mg/kg	<30 mg/kg
Arsenic	<3 mg/kg	<3 mg/kg	<3 mg/kg
Cadmium	<0.5 mg/kg	<0.5 mg/kg	<0.5 mg/kg
Mercury	<0.5 mg/kg	<0.5 mg/kg	<0.5 mg/kg
Lead	<5 mg/kg	<5 mg/kg	<5 mg/kg
Mycotoxins	NEG	NEG	NEG

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 **lipase** producing strain highlighted in red.

The safety of the **lipase** enzyme is fully supported by repeated testing of the **lipase** and other enzymes produced by members of this Safe Strain Lineage. The blue coloured boxes indicate strains for which we conducted toxicology studies. The NOAEL for this **lipase** 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

Appendix 5: External Expert Opinion Letter from Dr. Michael Pariza

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7102 Valhalla Trail
Madison, WI 53719
(608) 271-5169
mwpariza@gmail.com

Michael W. Pariza, Member

July 19, 2018

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 Lipase 3 enzyme preparation from *Aspergillus tubingensis* that is expressed in a non-pathogenic, non-toxigenic strain of *Trichoderma reesei*

Dear Dr. Sewalt,

I have reviewed the information you provided on DuPont's Lipase 3 enzyme preparation, which is produced by *Trichoderma reesei* Morph Lip3 (GICC03373), a production strain that has been genetically modified to over-express the native Lipase 3 from *Aspergillus tubingensis* (DuPont IB strain 1M341). The intended uses of Lipase 3 are as a processing aid in brewing, baking, cereal beverage manufacture, pasta production, potable alcohol, and fuel ethanol manufacture with the resulting co-products (such as distillers grains, corn gluten/meal, and distillers corn oil) destined for animal food, where the enzyme is either not present in the final food, or present at trace levels as inactive protein having no function or technical effect.

In evaluating Lipase 3, I considered the biology of *T. reesei* and *A. tubingensis* and their history of safe use in food-grade enzyme manufacture; safety evaluation studies on the Lipase 3 enzyme preparation; safety evaluation studies on other food grade enzymes expressed by DuPont's safe lineage of *T. reesei* production strains; history of safe use in foods of lipases from other microbial species; information that you provided regarding the safe lineage of the production organism, cloning methodology, manufacturing materials and procedures, and product specifications; and information that is publically available in the peer-reviewed scientific literature.

By way of background, *T. reesei* is used widely by enzyme manufacturers worldwide for the production of enzyme preparations that are, in turn, used in human food, animal feed, and numerous industrial enzyme applications. DuPont's lineage of safe *T. reesei* production

strains, including *T. reesei* Morph Lip3 (GICC03373), was derived through a series of modifications from *T. reesei* QM6a, the original non-pathogenic and non-toxigenic wild-type parental strain used to produce this safe lineage of *T. reesei* enzyme production strains. Published literature, government laws and regulations, for example FR 64:28658-28362 (1999), reviews by expert panels such as FAO/WHO JECFA (1992), and DuPont's (legacy Genencor and Danisco) unpublished safety studies, all support the conclusion that the lineage to which these production strains belong is safe and suitable for use in the manufacture of food-grade and feed-grade enzymes.

Strains within this safe lineage are used to manufacture many food and feed enzymes, including chymosin, transglucosidase, cellulases, glucoamylase, α-amylase, β-glucosidase/cellulase, acid fungal protease, α-glucosidase, lipase, phytase, trehalase, 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, 567 and 727, all of which carry the decision statement, "FDA has no questions."

Aspergillus tubingensis is virtually indistinguishable from *Aspergillus niger* and is often referred to as *Aspergillus niger* var. *tubingensis*. The organism is classified within the '*Aspergillus* section Nigri', a species/subspecies group of closely related microorganisms that are widely used by enzyme manufacturers worldwide for the production of enzyme preparations that are used in human food, animal feed, and numerous other industrial enzyme applications.

Lipases from a number of microbial sources have long histories of safe use in food and feed manufacture. The Lipase 3 enzyme gene that was cloned into the *T. reesei* Morph Lip3 (GICC03373) production strain was obtained from *A. tubingensis* (DuPont IB strain 1M341). The Lipase 3 enzyme protein has been sequenced and studied for potential safety issues, specifically amino acid sequences that might elicit allergenicity or toxicity concerns. No such sequences were found.

The Lipase 3 enzyme preparation was evaluated for acute and inhalation toxicity in Wistar rats, dermal irritation in rabbits and mice, eye irritation in rabbits, genotoxicity in a number of test systems, and subchronic toxicity in a 90 day oral gavage study in Wistar rats. No dose-related adverse events were observed in any of these studies. The NOAEL for the Lipase 3 enzyme preparation was established as the highest dose tested in to 90 day oral gavage study, 160.6 mg total protein/kg bw/day equivalent to 123.15 mg TOS kg bw/day. The maximum (worst case) cumulative exposure for consumers to Lipase 3 from brewing processes and baking was calculated as 0.410 mg TOS/kg bw/day, giving a Margin of Safety for Lipase 3 from all uses of 300. For cattle, pigs, and poultry, respectively, the Margin of Safety calculations from all uses combined are 319, 255, and 294, respectively. All of these values (for humans and target animal species) are well above the traditionally accepted Margin of Safety of 100 for food ingredients.

The cloning techniques and methodologies employed to construct *T. reesei* Morph Lip3 (GICC03373) are appropriate for use in the genetic modification of production strains for food ingredient manufacture. In addition, the manufacturing process including the ingredients used for fermentation, extraction and concentration of Lipase 3, and the specifications for the Lipase 3 enzyme preparation, are appropriate for a food ingredient.

Based on the foregoing, I concur with the evaluation made by DuPont that the *T. reesei* Morph Lip3 (GICC03373) production strain is safe and appropriate to use for the manufacture of food-grade Lipase 3. I further conclude that the Lipase 3 enzyme preparation, manufactured in a manner that is consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food-grade specifications, is GRAS (Generally Recognized As Safe) for use as a processing aid in brewing, baking, cereal beverage manufacture, pasta production, potable alcohol, and fuel ethanol manufacture with the resulting co-products (such as distillers grains, corn gluten/meal, and distillers corn oil) destined for animal food, where the enzyme is either not present in the final food, or present at trace levels as inactive protein having no function or technical effect

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,

Michael W. Pariza

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

DuPont Industrial Biosciences
925 Page Mill Road
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April 3, 2019

To: Lane A. Highbarger, Ph.D.
Microbiology and Regulatory Review
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

From: Vincent J. Sewalt, Ph.D.
Senior Director, Product Stewardship & Regulatory
Danisco US Inc.
(operating as DuPont Industrial Biosciences)

Re: GRAS Notice No. GRN 000808 – *Aspergillus tubingensis* lipase enzyme preparation produced by *Trichoderma reesei*

Dear Dr. Highbarger:

Thank you for your review of our submission. We are providing this letter in response to FDA's request for information that was sent via email on April 2, 2019 regarding the *Aspergillus tubingensis* lipase enzyme preparation produced by *Trichoderma reesei*. We have copied your information requests above each of our responses for ease of reference:

1. **Is the enzyme secreted into the culture media or do you lyse/break the cells in some manner?**

   The lipase is secreted into the culture media.

DuPont Industrial Biosciences
925 Page Mill Road
Palo Alto, CA. 94304

2. **You have given us the sequence; would you please provide use with the molecular weight of the expressed protein.**

   The molecular weight of the mature lipase is 28.9 kDa.

If you have any further questions regarding GRN 000808, please do not hesitate to contact us.

Sincerely,

[redacted]

Vincent J. Sewalt, Ph.D.
Senior Director, Product Stewardship & Regulatory
Danisco US Inc.
(operating as DuPont Industrial Biosciences)