Alpha-Galactosidase Enzyme Produced by a Genetically Modified *Aspergillus niger*

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PART 1- SIGNED STATEMENT AND CERTIFICATION

Signed statement of the conclusion of GRAS (Generally Recognized as Safe) and certification of conformity to 21 CFR §170.205-170.260

§170.225(c)(1) – Submission of GRAS notice:

Novozymes North America Inc. is hereby submitting a GRAS (Generally Recognized as Safe) notice in accordance with subpart E of part 170.

§170.225(c)(2) - The name and address of the notifier:

Novozymes North America Inc.
77 Perry Chapel Church Rd.,
Box 576 Franklinton, NC 27525

§170.225(c)(3) – Appropriately descriptive term:

The appropriately descriptive term for this notified substance is alpha galactosidase enzyme preparation produced by genetically modified *Aspergillus niger.*

§170.225(b) – Trade secret or confidential:

This notification does not contain any trade secret or confidential information.

§170.225(c)(4) – Intended conditions of use:

The alpha galactosidase enzyme preparation is used as a processing aid during food manufacturing to catalyze the hydrolysis of terminal, non-reducing α-D-galactose residues in α-D-galactosides, including galactose oligosaccharides, galactomannans and galactolipids. The food applications where this alpha galactosidase will be used is in processing of carbohydrates in order to release sugars from oligosaccharides present in raw materials. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following Good Manufacturing Practices. The "general" population is the target population for consumption.

§170.225(c)(5) - Statutory basis for GRAS conclusion:

This GRAS conclusion is based on scientific procedures.

§170.225(c)(6) – Premarket approval:

The notified substance is not subject to the premarket approval requirements of the FD&C Act based on our conclusion that the substance is GRAS under the conditions of the intended use.

§170.225(c)(7) – Availability of information:

This notification package provides a summary of the information which supports our GRAS conclusion of the notified substance. Complete data and information that are the basis for this GRAS conclusion is available to the Food and Drug Administration for review and copying during customary business hours at Novozymes North America, Inc. or will be sent to FDA upon request.

§170.225(c)(8) - FOIA (Freedom of Information Act):

Parts 2 through 7 of this notification do not contain data or information that is exempt from disclosure under the FOIA (Freedom of Information Act).

§170.225(c)(9) – Information included in the GRAS notification:

To the best of our knowledge, the information contained in this GRAS notification is complete, representative, and balanced. It contains both favorable and unfavorable information, known to Novozymes and pertinent to the evaluation of the safety and GRAS status of the use of this substance.

Carissa Lannon Date: December 9, 2022
Regulatory Affairs Specialist I

PART 2 - IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS AND PHYSICAL OR TECHNICAL EFFECT OF THE NOTIFIED SUBSTANCE

2.1 IDENTITY OF THE NOTIFIED SUBSTANCE

The subject of this notification is an alpha galactosidase enzyme preparation produced by submerged fermentation of a non-toxigenic, non-pathogenic, genetically modified strain of *Aspergillus niger* microorganism carrying the gene coding for alpha galactosidase from *Aspergillus niger*.

Key enzyme and protein chemical characteristics of the alpha galactosidase are given below:

Classification:	Alpha galactosidase
Systemic Name:	alpha-D-galactoside galactohydrolase
Accepted Name:	Alpha galactosidase
EC No.:	3.2.1.22
CAS No.:	9025-35-8
Molecular Wt:	88 kDa
Specificity:	Hydrolysis of terminal, non-reducing α-D-galactose residues in α-D-galactosides, including galactose oligosaccharides, galactomannans and galactolipids
Amino acid sequence:	the total nucleotide and amino acid sequences have been determined

2.2 IDENTITY OF THE SOURCE

2.2(a) Production Strain

The *Aspergillus niger* production strain, designated 1633-C4645-3, was derived via the recipient strain, C4645, from a natural isolate of *Aspergillus niger* strain C40. The strain has been deposited in a recognized culture collection.

The alpha-galactosidase expression plasmid used in the strain construction contains strictly defined chromosomal DNA fragments and synthetic DNA linker sequences. The DNA sequence for the introduced gene is based on the *aglC* sequence encoding alpha-galactosidase from *Aspergillus niger*.

This genetically modified production organism complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large-Scale Practice) microorganisms (1). It also meets the criteria for a safe production microorganism as described by Pariza and Foster (2) and later Pariza and Johnson (3) and several expert groups (1) (4) (5) (6) (7) (8).

2.2(b) Recipient Strain

The recipient strain used in the construction of the alpha galactosidase production strain was modified at several chromosomal loci during strain development to inactivate genes encoding several amylases and proteases. Furthermore, the fumonisin gene cluster and the oxaloacetate hydrolase gene were deleted, together with the deletion of additional genes encoding unwanted

proteins that can be preset in the culture supernatant. The lack of the above compounds and proteins represents improvements in the product purity, safety, and stability.

2.2(c) Alpha Galactosidase Expression Plasmid

The alpha galactosidase expression plasmid used to introduce the *aglC* gene in the recipient strain is based on the replication origin of *Escherichia coli*. No fragments of the vector backbone are introduced into the production strain. The plasmid contains the expression cassette consisting of an *Aspergillus niger* promotor, the *aglC* sequence encoding alpha galactosidase, a transcriptional terminator from *Aspergillus niger* and a selective marker, *amdS* (*acetamidase*), derived from *Aspergillus nidulans*.

Only the expression cassette with elements between the promoter fragment and the terminator are present in the final production strain. This has been confirmed by Southern blot analysis and PCR analysis followed by DNA sequencing.

2.2(d) Construction of the Recombinant Microorganism

The production strain, *Aspergillus niger* 1633-C4645-3, was constructed from the recipient strain through the following steps:

1) The expression cassette from plasmid pHUda1633 was integrated into four specific loci in recipient strain by targeted homologous recombination to these loci.

2) The selection of transformants was achieved by growing on a minimal medium and subsequent screening for expression of the alpha-galactosidase

The resulting production strain containing one copy of the *aglC* gene at each of the four loci was named 1633-C4645-3.

Sequence confirmation of the inserted expression cassettes and the flanking regions at the integration loci was performed in the production strain.

2.2(e) Stability of the Introduced Genetic Sequences

The DNA is integrated into the *Aspergillus niger* chromosome. Thus, it is poorly mobilized for genetic transfer to other organisms and is considered mitotically stable. The phenotypic and genetic stability of the *Aspergillus niger* is proven by its capacity to produce a constant level of the alpha galactosidase enzyme. This was assessed by measuring the enzyme activity in three independent batches of the food enzyme, as outlined in Table 2. Furthermore, the protein spectrum for three batches showed identical expression profiles. Thus, stable enzyme production of the desired enzyme combined with the identical protein expression profile confirm the stability of the *Aspergillus niger* production strain.

2.2(f) Antibiotic Resistance Gene

No functional antibiotic resistance genes were left in the strain as a result of the genetic modifications. The absence of these genes was verified by genome sequence analysis.

2.2(g) Absence of Production Organism in Product

The absence of the production organism is an established specification for the commercial product. The production organism does not end up in food and therefore the first step in the safety assessment as described by IFBC is satisfactorily addressed (4).

2.3 METHOD OF MANUFACTURE

The quality management system used in the manufacturing process for the enzyme preparation complies with the requirements of ISO 9001 and is produced under a standard manufacturing process in accordance with current Good Manufacturing Practices using ingredients that are accepted for general use in foods, and under conditions that ensure a controlled fermentation (9) (10) (11).

The enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in the Food Chemicals Codex (12). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (13).

2.3(a) Raw Materials

The raw materials used in the fermentation and recovery process for the enzyme concentrate are standard ingredients used in the enzyme industry (9) (10) (11). The raw materials conform to Food Chemicals Codex specifications except those raw materials which do not appear in the FCC (12). For those not appearing in the FCC, internal specifications have been made to ensure suitability and acceptability for use in food enzyme production. As part of the overall Quality program, Novozymes Quality Department follows a raw material approval program to qualify and approve suppliers. Raw materials are purchased only from approved suppliers and are verified upon receipt.

Any antifoams or flocculants used in fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 10, 1998. The maximum use level of the antifoams and/or flocculants, if used in the product, is not greater than 1%.

2.3(b) Fermentation Process

The alpha galactosidase is produced by pure culture, submerged, fed-batch fermentation of a genetically modified strain of *Aspergillus niger* as described in Part 2.

During fermentation, the alpha galactosidase enzyme that is produced by *Aspergillus niger* is secreted into the fermentation media.

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 done to ensure absence of foreign microorganisms and confirm strain identity.

2.3(c) Production Organism

Each batch of the fermentation process is initiated with a stock culture of the production organism, *Aspergillus niger*. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

2.3(d) Criteria for the Rejection of Fermentation Batches

Growth characteristics during fermentation are observed both macroscopically and microscopically. Samples are taken from both the seed fermenter and the main fermenter before inoculation, at regular intervals during cultivation and before transfer/harvest. These samples are tested for microbiological contamination by microscopy and by plating on a nutrient agar followed by a 24-48-hour incubation period.

The fermentation is declared "contaminated" if one of the following conditions are fulfilled:

1) Contamination is observed in 2 or more samples by microscopy

2) Contamination is observed in two successive agar plates at a minimum interval of 6 hours

Any contaminated fermentation is rejected.

2.3(e) Recovery Process

The recovery process is a multi-step operation designed to separate the desired enzyme from the microbial biomass and partially purify, concentrate, and stabilize the enzyme.

2.3(f) Purification Process

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

1) Pretreatment - pH adjustment and flocculation

2) Primary Separation – vacuum drum filtration or centrifugation

3) Concentration - ultrafiltration and/or evaporation

4) Pre- and Germ Filtration - for removal of residual production strain organisms and as a general precaution against microbial degradation

5) Final concentration – evaporation and/or ultrafiltration

6) Preservation and Stabilization of the liquid enzyme concentrate

The enzyme concentrate is stabilized with sorbitol and glycerol. The liquid product is further formulated by the addition of water and preserved with potassium sorbate and sodium benzoate. See Table 1 below.

2.4 COMPOSITION AND SPECIFICATIONS

The final products are analyzed according to the specifications given below.

2.4(a) Quantitative Composition

Table 1 below identifies the substances considered as diluents, stabilizers or preservative raw materials used in the enzyme preparation. The fermentation media used in the manufacturing of the alpha galactosidase enzyme preparation does not contain any major food allergens.

Table 1. Typical composition raw materials of the enzyme preparations

Substance	Approximate Percentage
Enzyme Solids (TOS*)	12.2%
Glycerol	>50%
Sorbitol	30-45%
Water	5-25%
Sodium Benzoate	<0.5%
Potassium Sorbate	<0.5%

**Total Organic Solids: defined as: 100% - water – ash.

2.4(b) Specifications

The alpha galactosidase enzyme preparation complies with the recommended purity specification criteria for "Enzyme Preparations" as described in *Food Chemicals Codex* (12). In addition, it also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications (13).

This is demonstrated by analytical test results of three representative enzyme batches. See Table 2 below.

Table 2. Analytical data for three food enzyme batches.

Parameter	Specifications	PPI47339	PPI48957	PPI50655
Alpha Galactosidase activity	GALU(N)DV/g	854	881	804
Total viable count	$\leq 10^4$/g	100	<100	<100
Lead	<5 mg/kg	<0.5	<0.5	<0.5
Salmonella sp.	ND in 25g of sample	ND	ND	ND
Total coliforms	≤30/g	< 4	< 4	< 4
Escherichia coli	ND in 25 g of sample	ND	ND	ND
Antimicrobial activity	ND	ND	ND	ND
Production Organism	ND	ND	ND	ND

*ND: Not Detected
**LOD: Limit of Detection

2.5 PHYSICAL OR TECHNICAL EFFECT

2.5(a) Mode of Action

The active enzyme is an alpha galactosidase (EC 3.2.1.22). The food applications where this alpha galactosidase will be used is in hydrolyzing of raffinose family oligosaccharides (RFOs) found in pulses that contain α-1,6-glycosidic bonds, into simple sugars and sucrose (14).

The function of alpha galactosidases is mainly to reduce oligosaccharides into mono- and di-saccharides.

2.5(b) Intended Use

Alpha galactosidase enzymes are used as processing aids in a wide range of food products (15) (16) (17) (18). The food applications where this alpha galactosidase will be used is in plant-based yogurt and dairy analogues, as well as plant-based meat analogues.

For plant-based yogurt and dairy analogues, alpha galactosidase is used as a processing aid in the production of legume milk, such as soy milk. Once the soy base is ready, it is further processed into plant-based yogurt and dairy analogues in processed food and beverages, such as soy milk, soy drink, yogurt, and ice cream. This application is not limited to soymilk and can be applied to a non-exhaustive list of legumes, such as pea and lentils. Because the function of alpha galactosidase is to reduce oligosaccharides into mono- and di- saccharides, Novozymes used the sugar content of soy milk in the dietary exposure calculations. According to the USDA FoodData Central, 3.5 grams of sugar is found in 100 grams of soymilk (19).

For plant-based meat analogues, alpha galactosidase is applied as a processing aid during the pre-treatment step in the extrusion process to hydrolyze RFOs into simple sugars and sucrose. Alpha galactosidase is denatured by heat during extrusion cooking and therefore does not exert a function in the final food (20) (21). Plant-based meat analogue products may be made by extrusion of legumes, e.g., soy, wheat, and pea. Using the USDA FoodData Central, Novozymes search for the highest sugar content between soy, wheat, and pea to be used in the dietary exposure calculations:

1) Soybean sugar content: 7.33 grams of sugar found in 100 grams of soybeans (22).
2) Wheat sugar content: 0.27 grams of sugar found in 100 grams of wheat (23).
3) Pea sugar content: 5.67 grams of sugar found in 100 grams of pea (24).

2.5(c) Use Levels

Food enzyme preparations are used by food manufacturers according to the Quantum Satis (QS) principle i.e., at a level not higher than the necessary dosage to achieve the desired enzymatic reaction in accordance with Good Manufacturing Practices (GMP).

The dosage applied in practice by a food manufacturer depends on the process. The initial recommendation by the enzyme manufacturer is only the starting point for the food producer and is optimised by the manufacturer of the food to fit the process conditions.

From a technological position, there are no “normal or maximum use levels”. But a food producer who would add much higher doses than what is needed would experience untenable costs as well as negative technological consequences.

The maximum recommended use levels for plant-based yogurt and dairy analogues in processed food and beverages are 17,634 GALU(N) per kg soybeans.

The maximum recommended use levels for plant-based meat analogues in processed food are 3,291 GALU(N) per kg plant protein.

2.5(d) Enzyme Residues in the Final Food

The alpha galactosidase enzyme preparation is used during processing and does not exert any enzymatic activity in the final food. This is due to a combination of various factors and depends on the process conditions used by the individual food producer. These factors include denaturation of the enzyme during heat processing, depletion of the substrate, physical removal of the enzyme, etc. In most cases, a heat treatment step is part of the manufacturing process for production of food ingredients and this process will be enough to inactivate or denature the enzyme protein.

Consequently, the presence of residues of food enzymes in the final food does not lead to any effect in or on the final food. The enzyme action has taken place during the food manufacturing process and is complete before the food product is available for delivery to consumers.

PART 3 - DIETARY EXPOSURE

To provide a "worst case" scenario for the calculation of the possible daily human exposure, an assumption was made that all the enzyme product is retained in the final food product. The general population is the target population for consumption. There is no specific subpopulation.

3(a) Assumptions in Dietary Exposure

Overall, the human exposure to the alpha galactosidase will be negligible because the enzyme preparation is used as a processing aid and generally at lower dosages.

The food enzyme is used in the manufacture of a wide variety of foods, food ingredients and beverages. Due to this wide variety of applications, the most appropriate way to estimate the human consumption in the case of food enzymes is using the Budget Method (25) (26).

The total TMDI represents a highly exaggerated value because of (among others) the following reasons:

- It is assumed that ALL producers for all processed food use the food enzyme at the highest recommended level.
- For the calculation of the TMDI in food as well as in beverage, the TOS for each application was combined and the total sum was used as the factor for the TMDI in the MOS (margin of safety) calculation.
- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed daily over the course of a lifetime.

Using these assumptions, the enzyme preparation will be consumed by humans at the maximum recommended dose for all applications. and will provide a highly conservative margin of safety.

Also, the consumption is further exaggerated since the enzyme protein and the other substances resulting from the fermentation are diluted or removed in certain processing steps. Therefore, the safety margin calculation derived from this method is highly exaggerated.

The alpha galactosidase enzyme preparation has an average activity of 846 GALU(N) per gram and approximately 12.2% TOS (Total Organic Solids) content. This corresponds to an activity/TOS ratio of 6.93 GALU(N) per mg TOS.

Using the highest enzyme preparation dosage applied is 17,634 GALU(N) per kg of soybeans which corresponds to 2544 mg TOS per kg soybeans.

3(b) Food Consumption Data

Assumptions in the Budget Method

Solid Food:

The maximum energy intake over the course of a lifetime is 50 kcal/kg body weight (b/w) /day. 50 kcal corresponds to 25 g food. Therefore, adults ingest 25 g food per kg body weight per day.

Assuming that 50% of the food is processed food, the daily consumption of processed food will be 12.5 g processed foods per kg body weight.

Under the assumption that all plant-based yogurt and dairy analogues and plant-based meat analogues contain a maximum of 7.33g sugar per 100g of soybeans (22), the intake 12.5 g processed food/kg bw/day will correspond to a maximum intake of 12.5 g processed food/kg bw/day x 7.33 g sugar/ 100g soybeans = 0.92 g processed food/ kg bw/day.

Based on this, processed food will maximally contain 2.34 mg TOS/kg bw/day

Liquids:

The maximum intake of liquids (other than milk) is 100 mL/kg body weight (bw) day.

Assuming that 25% of the non-milk beverages have been treated with the enzyme preparation, the daily consumption will be 25 mL processed beverages per kg body weight.

Under the assumption that all processed plant-based dairy analogue beverages contain a maximum of 3.65g sugar per 100g of soy milk (19), the intake 25 mL processed beverages/kg bw/ day will correspond to a maximum intake of 25 mL processed beverages/kg bw/day x 3.65g sugar/100g soy milk = 0.91 g processed beverages/ kg bw/day.

Based on this, processed beverages will maximally contain 2.32 mg TOS/kg bw/day

It is assumed that the densities of the beverages are ~ 1.

TMDI calculation

To represent a worst-case scenario for the maximum human exposure value, it is assumed that foods represented for each application are consumed daily. The final TMDI is calculated taking the TOS value for all applications.

The total maximum daily intake (TMDI) of the enzyme is 4.66 mg TOS/kg bw/day.

Margin of Safety

The margin of safety is calculated as dose level with no adverse effect (NOAEL) divided by the estimated human consumption, TMDI. The safety margin calculation derived from this method is highly exaggerated.

The NOAEL dose level in the 13-week oral toxicity study in rats conducted on alpha galactosidase tox batch PPI47297 was the highest dosage possible, 1305 mg TOS/kg bw/day. See Table 3 below.

Table 3. Calculation of the Margin of Safety

NOAEL (mg TOS/kg bw/day)	1305
*TMDI (mg TOS/kg bw/day)	4.66
Margin of Safety	280

*based on the worst-case scenario

PART 4 - SELF-LIMITING LEVELS OF USE

This part does not apply

PART 5 - COMMON USE IN FOOD BEFORE 1958

This part does not apply

PART 6 - NARRATIVE ON THE CONCLUSION OF GRAS STATUS

The information provided in the following sections is the basis for our conclusion of the general recognition of safety for the alpha galactosidase enzyme preparation. The evaluation follows the generally recognized methodology and the decision tree by Pariza and Johnson 2001 (3). Our safety evaluation in Part 6 follows the approach described in the Enzyme Technical Association publication (27) which includes an evaluation of the production organism, the donor strain, the introduced DNA, the enzyme, and the manufacturing process. Data and information cited in this notification is generally available and Part 6 does not contain any data or information that is exempt from disclosure under the FOIA.

6(a) Pariza and Johnson Decision Tree Analysis

This alpha galactosidase enzyme preparation produced by *Aspergillus niger* was evaluated according to the decision tree published in Pariza and Johnson, 2001 (3). The result of the evaluation is presented below in the Decision Tree.

1. Is the production strain genetically modified?
   YES
   If yes, go to 2.
2. Is the production strain modified using rDNA techniques?
   YES
   If yes, go to 3.
3. Issues relating to the introduced DNA are addressed in 3a-3e.
   a. Does the expressed enzyme product which is encoded by the introduced DNA have a history of safe use in food?
      YES
      If yes, go to 3c.
   c. Is the test article free of transferable antibiotic resistance gene DNA?
      YES
      If yes, go to 3e.
   e. 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 products?
      YES
      If yes, go to 4.
4. Is the introduced DNA randomly integrated into the chromosome?
   NO
   If no, go to 6.
6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?
   YES
   Test article is accepted.

6(b) Safety of the Manufacturing Process

This Part describes the manufacturing process for the alpha galactosidase, which follows standard industry practices (9) (10) (11).

The quality management system used in the manufacturing process for the enzyme complies with the requirements of ISO 9001. It is manufactured in accordance with current Good Manufacturing Practices, using ingredients that are accepted for general use in foods, and under conditions that ensure a controlled fermentation.

The enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in the Food Chemicals Codex (12). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (13).

6(c) Safety of the Production Organism

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food (2) (3).

If the organism is non-toxigenic and non-pathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, are safe to consume (4). Pariza and Foster 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" (2).

Aspergillus niger has a long history of safe use in the production of industrial enzymes and chemicals of both food grade and technical grade. *Aspergillus niger* is listed as a production/donor organism for a series of food-grade carbohydrases, oxidoreductases, lipases, glucanotransferase, and proteases in published scientific literature (3).

Carbohydrase, pectinase, protease, glucose oxidase, catalase, lipase and lactase enzyme preparations from *Aspergillus niger* are included in the GRAS petition 3G0016 (filed April 12th, 1973) that FDA, on request from the Enzyme Technical Association (ETA), converted into separate GRAS Notices (GRN 89, 111, 132) (28). Based on the information provided by ETA, as well as the information in GRP 3G0016 and other information available to FDA, the agency did not question the conclusion that enzyme preparations from *Aspergillus niger* are GRAS under the intended conditions of use. Analogous conclusions were drawn in GRAS Notices GRN 651, 657, 699, 739, and others which all describe food enzymes produced by *Aspergillus niger* strains (28).

In 1997, *Aspergillus niger* became one of the ten microbial species/strains that were eligible for exemption under 40 CFR Part 725 as recipient microorganisms under the TSCA biotechnology regulations (29). Also, *Aspergillus niger* was reviewed and was concluded to be a safe source organism by Olempska-Beer et al. (30) and Schuster et al. (31) under Good Manufacturing Practice (GMP) and with mycotoxin testing.

An evaluation of this genetically modified production microorganism for the alpha galactosidase, embodying the concepts initially outlined by Pariza and Foster, 1983 (2) and further developed by IFBC in 1990 (4), the EU SCF in 1991 (5), the OECD in 1992 (1), ILSI Europe Novel Food Task Force in 1996 (8), FAO/WHO in 1996 (7), JECFA in 1998 (13) and Pariza and Johnson in 2001 (3) demonstrates the safety of this genetically modified production microorganism strain.

The enzyme preparation is free of DNA encoding transferable antibiotic resistance gene DNA. The introduced DNA is well characterized and safe for the construction of microorganisms to be used in the production of food grade products. The DNA is stably integrated into the chromosome at specific sites in the chromosome and the incorporated DNA is known not to encode or express any harmful or toxic substances.

Some *Aspergillus niger* strains can produce ochratoxin A (31), and the production of fumonisin B2 has also been shown in *Aspergillus niger* (32). Ochratoxin A and fumonisin B2 are the two mycotoxins of concern in terms of human and animal safety that can be produced by *Aspergillus niger* strains (33).

The BO-1 safe strain lineage was found to be unable to produce unwanted secondary metabolites (ochratoxin A and fumonisin B2) under conditions that are known to induce mycotoxin production in fungi.

Based on the information presented above it is concluded that the *Aspergillus niger* production strain is considered a safe strain for the production of the alpha galactosidase enzyme.

6(d) Safe Strain Lineage

The safety of this *Aspergillus niger* production strain was established following published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients (3) (4). The *Aspergillus niger* production strain is derived from a safe strain lineage that is comprised of production strains for enzyme preparations which have full toxicological safety studies (i.e., 13-week oral toxicity study in rats, Ames test and chromosomal aberration test or micronucleus assay).

Novozymes has used *Aspergillus niger* as a production strain for a variety of enzymes for decades. Table 4 below outlines some of Novozymes enzyme preparations produced by *Aspergillus niger* production strains within the safe strain lineage and the safety studies conducted on those enzyme concentrates.

Table 4: Safe Strain Lineage

Enzyme	EC No.	Predecessor strain [1]	Donor strain	Safety studies [2]
Glucoamylase	3.2.1.3	*Aspergillus niger* BO-1	None	Yes
Pectin lyase	4.2.2.10	*Aspergillus niger* BO-1	*Aspergillus niger*	Yes
Lysophospholipase	3.1.1.5	*Aspergillus niger* BO-1	*Aspergillus niger*	Yes
Triacylglycerol lipase	3.1.1.3	*Aspergillus niger* BO-1	*Candida antarctica*	Yes
Glucoamylase	3.2.1.3	*Aspergillus niger* JaL303	*Aspergillus niger*	Yes
Glucoamylase	3.2.1.3	*Aspergillus niger* JaL303	*Talaromyces emersonii*	Yes
Glucoamylase	3.2.1.3	*Aspergillus niger* C878	*Trametes cingulata*	Yes
Alpha-amylase	3.2.1.1	*Aspergillus niger* C878	*Rhizomucor pusillus*	Yes
Glucoamylase	3.2.1.3	*Aspergillus niger* C2218	*Gloeophyllum trabeum*	Yes
Glucoamylase (GRN 657)	3.2.1.3	*Aspergillus niger* C2218	Penicillum oxalicum	Yes
Triacylglycerol lipase (GRN 158)	3.1.1.3	*Aspergillus niger* C2218	*Candida antarctica*	Yes
Mannanase (GRN 739)	3.2.1.78	*Aspergillus niger* C2218	*Talaromyces leycettanus*	Yes
Phospholipase A (GRN 651)	3.1.1.32	*Aspergillus niger* C2948	*Talaromyces leycettanus*	Yes
Trehalase (GRN 699)	3.2.1.28	*Aspergillus niger* C2218	*Corynascus sepedonium* (*Myceliophthora sepedonium*)	Yes
Phospholipase	3.1.1.32	*Aspergillus niger* C2218	*Talaromyces bacillisporus*	Yes

Table 4. Novozymes products derived from *A. niger* strains where safety studies have been carried out.
[1] The predecessor strain shows strains in the GM construction pathway. [2] At least the following: *in vitro* test for gene mutations in bacteria (Ames); *in vitro* test for chromosomal aberration or *in vitro* micronucleus assay; 13-week sub chronic oral toxicity study in rats. The conclusions of these studies were in all cases favorable.

All toxicological studies concluded that the test preparations did not exhibit any toxic or mutagenic effect under the conditions of the test. These studies support the view that strains derived from the *Aspergillus niger* strain lineage can be used safely for the production of food enzymes.

The fact that no issues are observed in safety studies on different enzymes (e.g., amylases, protease, xylanase) produced by strains derived from a common predecessor (*Aspergillus niger*), strongly supports the safety of the *A. niger* strain lineage, independent of which enzyme is produced.

The *A. niger* production strain, described in this assessment, is genetically modified by rDNA techniques as discussed in Part 2. The expressed alpha galactosidase enzyme preparation is free of DNA encoding transferable antibiotic resistance gene DNA. The introduced DNA is well characterized and safe for the construction of microorganisms to be used in the production of food grade products. The DNA is stably integrated into the chromosome and the incorporated DNA is known not to encode or express any harmful or toxic substances. The procedures used to modify the host organism are well defined and commonly used. Therefore, the elements needed to establish a safe strain lineage as defined in Pariza and Johnson, 2001 (3) have been met.

Based on the information presented in Parts 6, it is concluded that the *Aspergillus niger* production strain is part of the safe strain lineage and is considered a safe strain for the production of the alpha galactosidase enzyme.

6(e) Safety of the Donor Organism

As noted above, it is the safety of the production strain that should be the primary concern when assessing the safety of an enzyme used for food.

The donor organism of the alpha galactosidase is *Aspergillus niger*. As indicated in Part 2 the introduced DNA is well defined and characterized. The introduced DNA does not code for any known harmful or toxic substances.

6(f) Safety of the Alpha Galactosidase Enzyme

A wide variety of enzymes are used in food processing (2) (3). Alpha galactosidases account for a significant share of the world enzyme market with major uses in the food and feed processing (34). Alpha galactosidases are widely distributed in nature and have been isolated from a variety of sources, such as microbes, plants, few lower animals, and humans (35)**.**The mode of action of alpha galactosidases is to catalyze the hydrolysis of terminal, non-reducing α-D-galactose residues in α-D-galactosides, including galactose oligosaccharides, galactomannans and galactolipids (34).

Alpha galactosidase enzymes are classified as carbohydrases (3). In 1973, the Enzyme Technical Association submitted a GRAS petition (GRASP 3G0016) to the FDA that included information and data on enzymes produced by *A. niger*. In 2001, the FDA converted that petition into three notifications: GRN 88, 89 and 90. GRN 89 includes carbohydrases from *A. niger*. The FDA had "No Questions" and agreed that carbohydrases from *A. niger* were generally recognized as safe under the condition of use. Additionally, in the U.S., carbohydrase enzyme preparations from *Aspergillus niger* have been codified under 21 CFR Part 173.120.

A literature search was performed in April 2022 for the periods 2000 to 2022 on alpha galactosidase, utilizing the database Web of Science. Novozymes reviewed the available abstracts and found no indication that that alpha galactosidase is associated with toxicity or other adverse effects in humans or animals and the findings did not contradict our determination of the general recognition of safety of the alpha galactosidase enzyme. Novozymes also conducted a sequence homology to known allergens and toxins. The results showed no indication of allergenic or toxigenic potential of the alpha galactosidase.

From the information provided above, it is apparent that alpha galactosidase enzymes have a long history of use in food processing and are safe for human consumption.

6(g) Allergenic/Toxigenic Potential of the Alpha Galactosidase Enzyme

The ingestion of a food enzyme protein is not considered a food allergy concern. This is based on the following considerations:

1) Enzymes have a long history of safe use in food, with no indication of adverse effects or reactions.

2) The majority of proteins are not food allergens. A wide variety of enzyme classes and structures are naturally present in plant and animal-based foods, and based on previous experience, food enzymes are not homologues to known allergens, which make it very unlikely that a new enzyme would be a food allergen.

3) Enzymes in foods are added in concentrations in the low range of part per millions. The enzyme is typically removed or denatured during food processing and denatured protein has been shown to be very susceptible to digestion in the gastro-intestinal system. Moreover, a wide range of naturally occurring food enzymes have been shown to be very labile in the gastro-intestinal system even in the native unprocessed form.

The above statements are further supported by the publication: "Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry" (Bindslev-Jensen *et al*, 2006) (36).

In order to further evaluate the possibility that the alpha galactosidase will cross-react with known allergens and induce a reaction in an already sensitized individual, a sequence homology to known allergens was assessed. Following the guidelines developed by FAO/WHO, 2001 (37) and modified by Codex Alimentarius Commission, 2009 (38) the alpha galactosidase was compared to allergens from the FARRP allergen protein database (http://allergenonline.org) as well as the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee (**http://www.allergen.org**).

A search for more than 35% identity in the amino acid sequence of the expressed protein using a window of 80 amino acids and a gap penalty was done and showed no matches. Alignment of the alpha galactosidase to each of the allergens and identity of hits with more than 35% identity over the full length of the alignment was analyzed. No homology was found between the alpha galactosidase and any of the allergens from the databases mentioned above. Also, a search for 100% identity over 8 contiguous amino acids was completed. Again, no homology was found.

A sequence homology of alpha galactosidase to known toxins was assessed based on the information present in the UNIPROT database. This database contains entries from SWISSPROT and TREMBL. The homology among the emerging entries was below 16.4% indicating that the homology to any toxin sequence in this database is random and very low.

On the basis of the available evidence, it is concluded that oral intake of alpha galactosidase produced by *Aspergillus niger* is not anticipated to pose any food allergenic or toxigenic concerns.

6(h) Safety Studies Conducted

Novozymes has repeatedly used the procedures outlined by Pariza and Johnson to evaluate enzymes derived from *Aspergillus niger* production strains (3). As described in Part 6(c), Novozymes has concluded, that strains within the safe strain lineage of *Aspergillus niger* pose no safety concerns. Table 4 lists the strains within this lineage, with many having corresponding GRNs on file with the FDA, where toxicological safety studies have been performed.

These toxicological studies include genotoxicity, cytotoxicity and general toxicity activities. The toxicology studies have produced consistent findings indicating that the test article (enzyme concentrate) did not exhibit any toxic or mutagenic effects under the conditions of the test, thus supporting the safety of the enzymes produced by *Aspergillus niger* strains that are within this lineage.

It is reasonable to expect and conclude that enzymes produced by *Aspergillus niger* strains within this safe strain lineage will show similar toxicological profiles and further supports our conclusion that *Aspergillus niger* strains are safe hosts for the expression of enzymes. To further support this conclusion and to meet data requirements in other countries, we have conducted the following toxicological studies on the substance, the subject of this notification, as described below (39) (40).

6(i) Description of the Test Article

This section describes the studies and analysis performed to evaluate the safety of the use of the alpha galactosidase enzyme preparation.

The following studies were performed on test batch PPI47297 with favorable results:

- Reverse Mutation Assay (Ames test)
- In vitro Human Lymphocyte Chromosome Aberration Assay
- 13-week oral toxicity study in rats

All toxicology studies performed on the alpha galactosidase enzyme concentrate, batch PPI47297 from the *Aspergillus niger* production strain, were carried out in accordance with current OECD guidelines and in compliance with the OECD principles of Good Laboratory Practice (GLP).

These tests are summarized in Appendix 1. Based on the presented toxicity data and the history of safe use for the strain it can be concluded that the test preparation, represented by batch PPI47297 exhibits no toxicological effects under the experimental conditions described.

6(j) Results and Conclusion

The enzyme industry has performed hundreds of toxicology studies using a variety of enzymes (e.g. alpha galactosidases, amylases, glucanases, lipases etc.) derived from multiple production organisms (e.g. *Aspergillus niger*, *Trichoderma reesei, Bacillus subtilis* etc.) with no adverse findings observed in the conducted studies (3) (41) (42).

Results of the toxicity and mutagenicity tests described in Appendix 1 showed no toxicity or mutagenicity of the test article; enzyme concentrate batch PPI47297 produced by *Aspergillus niger.*

A critical review and evaluation of the alpha galactosidase enzyme preparation (subject of this notification) was done following the concepts of the Pariza papers and the recently described process for the evaluation of GRAS for industrial microbial enzymes by Sewalt et al. (2) (3) (27).

Based on the published, publicly available scientific information about *Aspergillus niger* production strains and alpha galactosidase enzymes used in food processing, along with the supporting data generated by Novozymes and using the decision tree evaluation method outlined by Pariza and Johnson (3), Novozymes considers the alpha galactosidase enzyme preparation (subject of this notification), produced by the *Aspergillus niger* production organism to be generally recognized as safe.

PART 7 – SUPPORTING DATA AND INFORMATION

All information indicated in the List of Appendices and References is generally available

APPENDICES

1. Summary of Toxicity Data, Alpha Galactosidase, Batch PPI47297, from *Aspergillus niger*, April 26, 2018, File No. 2018-05826-01.

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SUMMARY OF TOXICITY DATA

Alpha galactosidase, batch PPI47297, from *Aspergillus niger*

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1. ABSTRACT

The below series of toxicological studies were undertaken to evaluate the safety of Alpha galactosidase, batch PPI47297.

All studies were carried out in accordance with current OECD guidelines and in compliance with the OECD principles of Good Laboratory Practice (GLP). The studies were performed at Envigo (UK), Covance (UK) and Triskelion (NL) during the period June 2017 to April 2018.

The main conclusions of the studies can be summarized as follows:

- Alpha galactosidase, batch PPI47297, did not induce gene mutations in the Ames test, in the absence or presence of a rat liver metabolic activation system (S-9).
- Alpha galactosidase, batch PPI47297, did not induce micronuclei in cultured human peripheral blood lymphocytes following treatment in the absence and presence of a rat liver metabolic activation system (S-9).
- Alpha galactosidase, batch PPI47297 was non-irritant when evaluated using the *ex vivo/in vitro* eye irritation test using isolated chicken eye (ICE). Based on results obtained in the study, Alpha galactosidase, batch PPI47297 was considered as non-irritant (UN-GHS No Category).
- Alpha galactosidase, batch PPI47297 was evaluated using the *in vitro* skin irritation test (Epiderm) using reconstituted human skin membranes. Based on the results obtained in the study, Alpha galactosidase, batch PPI47297 was considered as non-irritant (UN GHS No Category).
- In a 13-week oral toxicity study in rats Alpha galactosidase, batch PPI47297, was well tolerated and did not cause any toxicologically significant changes at any dose level applied. The no-observed-adverse-effect level (NOAEL) was considered to be 1305.0 mg TOS/kg body weight/day, or an enzyme activity of 5360.9 GALU(N)DV/kg body weight/day.

Based on the present toxicity data it can be concluded that Alpha galactosidase, represented by batch PPI47297, exhibits no significant toxicological effects under the experimental conditions described.

2. TEST SUBSTANCE

The test substance is an Alpha galactosidase (E.C. 3.2.1.22).

2.1 Characterization

The toxbatch Alpha galactosidase, batch PPI47297, was used for the conduct of all the toxicological studies. The characterization data of the toxbatch is presented in Table 1.

Table 1. Characterization data of Alpha galactosidase, batch PPI47297

Batch number	PPI47297
Activity	513.5 GALU(N)DV/g
N-Total (% w/w)	1.19
Water (KF) (% w/w)	87.2
Dry matter (% w/w)	12.8
Ash (% w/w)	<0.3
Total Organic Solids (TOS[1]) (% w/w)	12.5
Specific gravity (g/mL)	1.044

[1] % TOS is calculated as 100% - % water - % ash - % diluents.

3. MUTAGENICITY

3.1 Bacterial Reverse Mutation assay (Ames test)

Alpha galactosidase, batch PPI47297 was assayed for mutation in four histidine-requiring strains (TA98, TA100, TA1535 and TA1537) of Salmonella typhimurium, and one tryptophan-requiring strain (WP2 uvrA pKM101) of Escherichia coli, both in the absence and presence of metabolic activation by an Aroclor 1254-induced rat liver post-mitochondrial fraction (S-9), in two separate experiments.

A 'treat and plate' procedure was used for all treatments in this study as Alpha galactosidase, batch PPI47297 is a high molecular weight protein (which may cause artefacts through growth stimulation in a standard plate-incorporation test).

All Alpha galactosidase, batch PPI47297 treatments in this study were performed using formulations prepared in water for irrigation (purified water), and all concentrations stated in this report include a correction to account for Total Organic Solids (TOS) content of 12.5% w/w, using a correction factor of 8.0.

Mutation Experiment 1 treatments of all the tester strains were performed in the absence and in the presence of S-9, using final concentrations of Alpha galactosidase, batch PPI47297 at 16, 50, 160, 500, 1600 and 5000 µg TOS/mL. Following these treatments, no evidence of toxicity was observed on the plates.

Mutation Experiment 2 treatments of all the tester strains were performed in the absence and in the presence of S-9. The maximum test concentration of 5000 µg TOS/mL was retained for all strains. Narrowed concentration intervals were employed covering the range 160-5000 µg TOS/mL, in order to examine more closely those concentrations of Alpha galactosidase, batch PPI47297 approaching the maximum test concentration and considered therefore most likely to provide evidence of any mutagenic activity. Following these treatments, no evidence of toxicity was observed on the plates.

The test article was completely soluble in the aqueous assay system at all concentrations treated, in each of the experiments performed.

Vehicle and positive control treatments were included for all strains in both experiments. The mean numbers of revertant colonies all fell within acceptable ranges for vehicle control treatments, and were elevated by positive control treatments.

Following Alpha galactosidase, batch PPI47297 treatments of all the test strains in the absence and presence of S-9, no notable and concentration-related increases in revertant

numbers were observed, and none that were ≥2-fold (in strains TA98, TA100 and WP2 uvrA pKM101) or ≥3-fold (in strains TA1535 and TA1537) the concurrent vehicle control. This study was considered therefore to have provided no evidence of any Alpha galactosidase, batch PPI47297 mutagenic activity in this assay system.

It was concluded that Alpha galactosidase, batch PPI47297 did not induce mutation in four histidine-requiring strains (TA98, TA100, TA1535 and TA1537) of Salmonella typhimurium, and one tryptophan-requiring strain (WP2 uvrA pKM101) of Escherichia coli when tested under the conditions of this study. These conditions included treatments at concentrations up to 5000 µg TOS/mL (the maximum recommended concentration according to current regulatory guidelines), in the absence and in the presence of a rat liver metabolic activation system (S-9) using a modified Treat and Plate methodology.

3.2 *In vitro* Micronucleus Test In Cultured Human Lymphocytes

Alpha galactosidase, batch PPI47297 was tested in an in vitro micronucleus assay using duplicate human lymphocyte cultures prepared from the pooled blood of two male donors in a single experiment. Treatments covering a broad range of concentrations, separated by narrow intervals, were performed both in the absence and presence of metabolic activation (S-9) from Aroclor 1254-induced rats. The test article was formulated in purified water and the highest concentration tested in the Micronucleus Experiment, 5000 µg total organic solids (TOS)/mL (an acceptable maximum concentration for in vitro micronucleus studies according to current regulatory guidelines) was determined following a preliminary cytotoxicity Range Finder Experiment.

Treatments were conducted (as detailed in the following summary table) 48 hours following mitogen stimulation by phytohaemagglutinin (PHA). The test article concentrations for micronucleus analysis were selected by evaluating the effect of Alpha galactosidase, batch PPI47297 on the replication index (RI). Micronuclei were analysed at concentrations from 3000 to 5000 µg/mL.

Appropriate negative (vehicle) control cultures were included in the test system under each treatment condition. The proportion of micronucleated binucleate (MNBN) cells in these cultures fell within current 95th percentile of the observed historical vehicle control (normal) ranges. Mitomycin C (MMC) and Vinblastine (VIN) were employed as clastogenic and aneugenic positive control chemicals respectively in the absence of rat liver S-9. Cyclophosphamide (CPA) was employed as a clastogenic positive control chemical in the presence of rat liver S-9. Cells receiving these were sampled in the Micronucleus Experiment at 24 hours (CPA, MMC) or 48 hours (VIN) after the start of treatment. All positive control compounds induced statistically significant increases in the proportion of cells with micronuclei.

All acceptance criteria were considered met and the study was therefore accepted as valid.

Treatment of cells with Alpha galactosidase, batch PPI47297 in the absence and presence S-9 resulted in frequencies of MNBN cells which were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle controls for the majority of all concentrations analysed (all treatments). The single exception to this was observed for the intermediate concentration analysed of 4000 µg TOS/mL following 24+24 hour treatment in the absence of S-9. However, this increase was small with a concentration mean MNBN cell frequency of 0.65% as compared to the concurrent vehicle control response of 0.25% which fell within the normal range of 0.1 to 0.84% MNBN cells. One of the two replicate cultures ('A' culture) did exhibit a slightly elevated MNBN cell frequency of 1.0% though this

was not observed in the 'B' culture or for any other test article treated culture at either higher or lower concentrations analysed. As such, this isolated statistical increase was not considered of biological relevance.

It is concluded that Alpha galactosidase, batch PPI47297 did not induce biologically relevant increases in micronuclei in cultured human peripheral blood lymphocytes following treatment in the absence and presence of an aroclor induced rat liver metabolic activation system (S-9). Concentrations were tested up to 5000 µg TOS/mL, a recommended regulatory maximum concentration for the in vitro micronucleus assay.

4. GENERAL TOXICITY

4.1 *Ex vivolin vitro* eye irritation test using Isolated Chicken Eye Test

The purpose of the study was to determine the eye irritation potential of Alpha galactosidase, batch PPI47297 in the Isolated Chicken Eye (ICE) test. The study was performed in accordance with the OECD guideline No. 438, adopted 26th July 2013.

Physiological saline was included as negative control and benzalkonium chloride (BAC) as positive control.

The chicken eyes were obtained from slaughter animals used for human consumption.

Three eyes were exposed to a single application of 30 µL neat test material for 10 seconds followed by 20 mL saline rinse. Corneal thickness (swelling), corneal opacity and fluorescein retention of damaged epithelial cells were evaluated. Moreover, histopathology of the corneas was performed.

Alpha galactosidase, batch PPI47297caused corneal effects consisting of very slight swelling (mean of 2%), slight opacity (mean score of 1.0) and very slight fluorescein retention (mean score of 0.5). Microscopic examination of the corneas generally revealed very slight erosion and very slight vacuolation of the epithelium in one cornea.

Applying the classification criteria for the ICE test, Alpha galactosidase, batch PPI47297is not considered to be an eye irritant, and hence no classification (Not classified both UN-GHS classification and EU-CLP classification).

4.2 *In vitro* skin irritation test using EpiDerm reconstituted skin membranes

The purpose of the study was to determine the *in vitro* skin irritation potential of Alpha galactosidase, batch PPI47297. The study was performed in accordance with the OECD guideline No. 439, adopted 28th July 2015.

The skin membranes were topically exposed to 30µL of the test substance for 60 minutes. The viability of the epidermal cells was assessed using MTT test at approx. 42 hours post-exposure. Negative and positive controls were run in parallel.

The principle for detection of viability via the MTT test is the conversion of the yellow tetrazolium salt (MTT) to the blue/purple product formazan by mitochondrial enzymes. The formation of formazan was measured using a spectrophotometer.

The mean viability of the skin membranes was 99 ± 3% compared to the negative control group.

Alpha galactosidase, batch PPI47297 was considered a non-irritant, and should not be classified (UN GHS No Category).

4.3 Toxicity Study by Oral Gavage Administration to Wistar Rats for 13 Weeks

The purpose of this study was to assess the systemic toxic potential of Alpha galactosidase, batch PPI47297 when administered orally to Han Wistar rats for 13 weeks. Three groups, each comprising 10 males and 10 females received doses of 10, 33 or 100% of Alpha galactosidase, batch PP147297 (equivalent to 130.5, 430.7 or 1305.0 mg TOS/kg/day or 536.1, 1769.1 or 5360.9 GALU(N)DV/kg/day). A similarly constituted control group received the vehicle (reverse osmosis water) at the same volume dose.

During the study, clinical condition, detailed physical examination and arena observations, sensory reactivity, grip strength, motor activity, body weight, food consumption, water consumption (by daily visual observation), ophthalmic examination, hematology (peripheral blood), blood chemistry, organ weight, macropathology and histopathology investigations were undertaken.

The general appearance and behaviour of the animals and sensory activity, grip strength and motor activity were unaffected by treatment and there were no treatment-related deaths. There was no effect of treatment on body weight gain or food and water consumption. There were no treatment related ophthalmoscopic, haematology, blood chemistry or urinalysis findings.

Organ weights were unaffected and there were no treatment-related macroscopic and microscopic findings.

It is concluded that oral administration of Alpha galactosidase, batch PPI47297 to Han Wistar rats at doses up to 100% of the test batch (equivalent to 1305.0 mg TOS/kg body weight/day, or an enzyme activity of 5360.9 GALU(N)DV/kg body weight/day) for 13 weeks was well tolerated, with no evidence of any adverse finding at any of the administered doses. Consequently, the no-observed-adverse-effect level (NOAEL) was considered to be 1305.0 mg TOS/kg body weight/day, or an enzyme activity of 5360.9 GALU(N)DV/kg body weight/day.

5. REFERENCES

5.1 Study reports

Triskelion: Study No. 10355/51; Novozymes Reference No.: 20176033: Evaluation of Eye Irritation Potential of Alpha-galactosidase, Batch PPI47297 *in vitro* using the Isolated Chicken Eye Test. (November 2017). LUNA file: 2017-17740.

Triskelion: Study No.: 25850/18; Novozymes Reference No.: 20176032: *In vitro* skin Irritation Test with Alpha Galactosidase, Batch PPI47297 using EpiDerm™ Reconstructed Skin Membranes. (September 2017). LUNA file 2017-13605.

Covance: Study No.: 8369679; Novozymes Reference No.: 20176026. Alpha galactosidaase, Batch PPI47297: Bacterial Reverse Mutation Assay using a Treat and Plate Modification. (January 2018). LUNA file: 2018-01808.

Covance: Study No.: 8369680; Novozymes Reference No.: 20176027: Alpha galactosidase, Batch PPI47297: *In vitro* Human Lymphocyte Micronucleus Assay. (November 2017). LUNA file: 2017-17447.

Envigo: Study No.: DJ33XJ; Novozymes Reference No.: 20176022: Alpha galactosidase, Batch PPI47297: Toxicity Study by Oral Gavage Administration to Han Wistar Rats for 13 Weeks. (April 2018). LUNA file: 2018-05824.

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE

Form Approved: OMB No. ; Expiration Date:
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
001120	Dec 9, 2022
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration, 5100 Paint Branch Pkwy., College Park, MD 20740-3835.

PART I – 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)*

3a. For New Submissions Only: Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd)*: _____

3b. 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)*: _____

PART II – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position
Janet Oesterling	Regulatory Affairs

Company *(if applicable)*: Novozymes North America

Mailing Address *(number and street)*: 77 Perrys Chapel Church Road

City	State or Province	Zip Code/Postal Code	Country
Franklinton	North Carolina	27525	United States of America

Telephone Number	Fax Number	E-Mail Address
252-915-1444		jao@novozymes.com

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

Name of Contact Person	Position

Company *(if applicable)*:

Mailing Address *(number and street)*:

City	State or Province	Zip Code/Postal Code	Country

Telephone Number	Fax Number	E-Mail Address

PART III – GENERAL ADMINISTRATIVE INFORMATION

1. Name of Substance

Alpha galactosidase enzyme preparation produced by a genetically modified Aspergillus niger

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

☒ Electronic Submission Gateway

☐ Paper

☐ Electronic files on physical media with paper signature page

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 FDA's files by reference? *(Check one)*

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

5. The submission incorporates by reference 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 determination of GRAS status *(Check one)*

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

7. Does the submission (including information that you are incorporating by reference) contain information that you view as trade secret or as confidential commercial or financial information?

☐ Yes *(Proceed to Item 8)*

☒ No *(Proceed to Part IV)*

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, see attached Designation of Confidential Information

☐ 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

PART IV – INTENDED USE

1. Describe the intended use of the notified substance including the foods in which the substance will be used, the levels of use in such foods, the purpose for which the substance will be used, and any special population that will consume the substance *(e.g., when a substance would be an ingredient in infant formula, identify infants as a special population)*.

Alpha galactosidase enzymes are used as processing aids in a wide range of food products. The food applications where this alpha galactosidase will be used is in processing of carbohydrates in order to release sugars from oligosaccharides present in raw materials. The maximum recommended use levels for plant-based yogurt and dairy analogues in processed food and beverages are 17,634 GALU(N) per kg soybeans. The intended population is the general adult population.

2. Does the intended use of the notified substance include any use in meat, meat food product, poultry product, or egg product? *(Check one)*

☐ Yes ☒ No

PART V – IDENTITY

1. Information about the Identity of the Substance

	Name of Substance[1]	Registry Used *(CAS, EC)*	Registry No.[2]	Biological Source *(if applicable)*	Substance Category *(FOR FDA USE ONLY)*
1	Alpha galactosidase	EC	3.2.1.22		
2					
3					

[1] Include chemical name or common name. Put synonyms *(whether chemical name, other scientific name, or common name)* for each respective item (1 - 3) in Item 3 of Part V *(synonyms)*

[2] Registry used e.g., CAS *(Chemical Abstracts Service)* and EC *(Refers to Enzyme Commission of the International Union of Biochemistry (IUB), now carried out by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB))*

2. Description

Provide additional information to identify the notified substance*(s)*, which may include chemical formula*(s)*, empirical formula*(s)*, structural formula*(s)*, quantitative composition, characteristic properties *(such as molecular weight(s))*, and general composition of the substance. For substances from biological sources, you should include scientific information sufficient to identify the source *(e.g., genus, species, variety, strain, part of a plant source (such as roots or leaves), and organ or tissue of an animal source)*, and include any known toxicants that could be in the source.

Alpha galactosidase with the molecular weight of 88 kDA. The general composition for the enzyme preparation is glycerol, sorbitol, water, enzyme solids, sodium benzoate and potassium sorbate. .Alpha galactosidases catalyze the hydrolysis of terminal, non-reducing α-D-galactose residues in α-D-galactosides, including galactose oligosaccharides, galactomannans and galactolipids. The function of alpha galactosidases is mainly to reduce oligosaccharides into mono- and di-saccharides.

3. Synonyms

Provide as available or relevant:

1	alpha-D-galactoside galactohydrolase
2	
3	

PART VI – OTHER ELEMENTS IN YOUR GRAS NOTICE

(check list to help ensure your submission is complete – check all that apply)

- ☒ Any additional information about identity not covered in Part V of this form
- ☒ Method of Manufacture
- ☒ Specifications for food-grade material
- ☒ Information about dietary exposure
- ☒ Information about any self-limiting levels of use *(which may include a statement that the intended use of the notified substance is not-self-limiting)*
- ☒ Use in food before 1958 *(which may include a statement that there is no information about use of the notified substance in food prior to 1958)*
- ☒ Comprehensive discussion of the basis for the determination of GRAS status
- ☒ Bibliography

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

PART VII – SIGNATURE

1. The undersigned is informing FDA that Novozymes North America *(name of notifier)* has concluded that the intended use(s) of Alpha galactosidase enzyme preparation produced by a genetically modified Aspergillus niger *(name of notified substance)* described on this form, as discussed in the attached notice, is (are) exempt from the premarket approval requirements of section 409 of the Federal Food, Drug, and Cosmetic Act because the intended use(s) is (are) generally recognized as safe.

2. ☒ Novozymes North America *(name of notifier)* agrees to make the data and information that are the basis for the determination of GRAS status available to FDA if FDA asks to see them.

Novozymes North America *(name of notifier)* 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.

77 Perrrys Chapel Church Road, Franklinton, NC 27525
(address of notifier or other location)

Novozymes North America *(name of notifier)* agrees to send these data and information to FDA if FDA asks to do so.

OR

☐ The complete record that supports the determination of GRAS status is available to FDA in the submitted notice and in GRP No. _____
(GRAS Affirmation Petition No)

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
Janet Oesterling Digitally signed by Janet Oesterling Date: 2022.12.09 11:28:11 -05'00'	Janet Oesterling, Regulatory Affairs Manager	12/09/2022

PART VIII – 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)
	GRASNotification_Alpha Galactosidase from Aspergillus niger_2022-08-30.pdf	Submission
	SummaryofToxicityData_AlphaGalactosidase_2018-04-26.pdf	Administrative

OMB Statement: Public reporting burden for this collection of information is estimated to average XX hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services,Food and Drug Administration, Office of Chief Information Officer, 1350 Piccard Drive, Room 400, Rockville, MD 20850. (Please do NOT return the form to this address.). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.