

PART 1: 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: Mannanase enzyme from *Talaromyces leycettanus* produced by *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 mannanase enzyme will be used as a processing aid during instant coffee production. 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.

(b) (6)



Janet Oesterling
Regulatory Affairs Specialist III

10/19/17
Date

**A Mannanase enzyme from *Talaromyces leycettanus* Produced by
*Aspergillus niger***

Janet Oesterling, Regulatory Affairs, Novozymes North America, Inc., USA

October 2017



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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 a mannanase enzyme produced by submerged fermentation of a genetically modified of *Aspergillus niger* microorganism carrying the gene coding for mannanase from *Talaromyces leycettanus*.

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

Systemic Name:	Mannan endo-1,4-beta-mannosidase
Accepted Name:	4-β-D-mannan mannanohydrolase; β-mannanase; endo-β-mannanase, mannanase
EC No.:	3.2.1.78
CAS No.:	37288-54-3
Specificity:	hydrolysis of (1->4)-beta-D-mannosidic linkages in mannans, galactomannans and glucomannans
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 272-C3085-10, was derived via the recipient strain, C3085, from a natural isolate of *Aspergillus niger* strain C40.

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 (4) (5) (1) (6) (7) (8).

The expression plasmid, used in the strain construction, plhar272, contains strictly defined chromosomal DNA fragments and synthetic DNA linker sequences. The DNA sequence for the introduced gene is based on the *manATL* sequence encoding a mannanase from *Talaromyces leycettanus*.

2.2(b) Recipient Strain

The recipient strain C3085 used in the construction of the production strain (*Aspergillus niger*) was modified at several chromosomal loci during strain development to inactivate genes encoding a number of amylases and proteases. Furthermore, the fumonisin gene cluster and the oxaloacetate hydrolase gene were deleted in C3085 together with the

deletion of additional genes encoding unwanted proteins that can be present in the culture supernatant. The lack of these represents improvements in the product purity, safety and stability.

2.2(c) Mannanase Expression Plasmid

The expression plasmid, plhar272, used to introduce the *manATL* gene in the recipient strain C3085 is based on the replication origin of *E. coli*. However, no fragments of the vector backbone are introduced in to the production strain. The plasmid contains the expression cassette consisting of a fragment of an *Aspergillus niger* promoter, the *manATL* sequence encoding the mannanase, a transcriptional terminator and the selective marker *amdS*.

The expression cassette and the *amdS* gene encoding an acetamidase are flanked by DNA regions used for targeted integration. Only this region is present in the final production strain. This has been confirmed by Southern blot and PCR analysis followed by DNA sequencing.

2.2(d) Construction of the Recombinant Microorganism

The production strain, *Aspergillus niger* (272-C3085-10), was constructed from the recipient strain C3085 through the following steps:

- 1) The expression cassette from plasmid plhar272 was integrated into four specific loci in strain C3085 by targeted homologous recombination to these loci. Targeted integration of the expression cassettes at these loci allows the expression of the mannanase gene (*manATL*) from the promoter.
- 2) The selection of transformants was achieved by growing on a minimal medium and subsequent screening for expression of mannanase.

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

The resulting Mannanase production strain containing one copy of the *manATL* gene at each of the four target loci was named 272-C3085-10.

2.2(e) Stability of the Introduced Genetic Sequences

The genetic stability of the introduced DNA sequences was determined by Southern blot hybridization. Analysis of samples from the end of production using the *manATL* gene specific probe showed an identical band pattern compared to the reference production strain (272-C3085-10) demonstrating the genetic stability of the introduced DNA during production. The transforming DNA is stably integrated into the *Aspergillus*

niger chromosome and as such is poorly mobilized for genetic transfer to other organisms and is mitotically stable.

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 (4) is satisfactorily addressed.

2.3 METHOD OF MANUFACTURE

This section describes the manufacturing process for the mannanase enzyme which follows standard industry practices (9) (10) (11). The quality management system used in the manufacturing process for the enzyme preparation 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. These methods are based on generally available and accepted methods used for production of microbial enzymes.

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. For those not appearing in the FCC, internal specifications have been made in line with FCC requirements. On arrival at Novozymes A/S, the raw materials are sampled by the Quality Control Department and subjected to the appropriate analyses to ensure their conformance to specifications.

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 mannanase enzyme preparation 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 enzyme 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*, described in Part 2. 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 (if required)
- 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 sucrose, sorbitol and sodium chloride. The preparation is 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

The mannanase enzyme preparation is sold in a liquid form. Table 1 below identifies the substances that are considered diluents, stabilizers, preservatives and inert raw materials used in the enzyme preparations. Also, the enzyme preparation, that is the subject of this notification, does not contain any major food allergens from the fermentation media.

Table 1. Typical compositions of the enzyme preparations

Substance	Approximate Percentage
Enzyme Solids (TOS*)	12%
Water	50 - 60%
Sucrose	20 - 30%
Sorbitol	1 – 10%
Sodium Chloride	1 - 5%
Sodium Benzoate	<0.5%
Potassium Sorbate	<0.5%

*Total Organic Solids, define as: 100% - water – ash – diluents.

2.4(b) Specifications

The mannanase 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	PPE42948	PPE43229	PPE47971
Mannanase activity	AMNU/g	27900	25700	27200
Total viable count	Upper limit 50,000	100	100	<100
Lead	Not more than 5 mg/kg	<0.5	<0.5	<0.5
Salmonella sp.	Absent in 25 g of sample	ND	ND	ND
Total coliforms	Not more than 30 per gr	< 4	< 4	< 4
Escherichia coli	Absent in 25 g of sample	ND	ND	ND
Antimicrobial activity	Not detected	ND	ND	ND
Ochratoxin A	<LOD mg/kg	<LOD	<LOD	<LOD
Fumonisin B ₂	<LOD mg/kg	<LOD	<LOD	<LOD

2.5 PHYSICAL OR TECHNICAL EFFECT

2.5(a) Mode of Action and Application

The active enzyme is mannanase (EC 3.2.1.78). The function of β -mannanases, as an endohydrolases, is to randomly cleave the 1,4- β -D mannan main chain of galactomannan, glucomannan, galactoglucomannan, and mannan (commonly referred to as hemicellulases) (14).

Mannans are present as either structural or storage carbohydrates and are a major, water-insoluble polysaccharide fraction of green coffee beans. Accordingly, the mannan present in the coffee bean causes high viscosity of the coffee extract when it

is concentrated before spray or freeze-drying. The reduction of viscosity, by cleaving the mannan portion with the mannanase enzyme, simplifies the production of instant coffee by improving the effectiveness with which the extracts can be concentrated and reduces energy costs of the drier (15).

Mannanase will be used as a processing aid during instant coffee production. The benefits of the action of the mannanase enzyme in the degradation of coffee beans is an increased extraction yield during instant coffee production.

2.5(b) Use Levels

The mannanase enzyme preparation will be added during the processing of instant coffee to reduce viscosity in the coffee extract. Commercial food enzyme preparations are generally used following the *Quantum Satis* (QS) principle, i.e. at a level not higher than the necessary dosage to achieve the desired enzymatic reaction and according to requirements for normal production following cGMP.

The dosage applied in practice by a food manufacturer depends on the process. It is based on an initial recommendation by the enzyme manufacturer and optimised to fit the process conditions.

The maximum recommended use level is 150 AMNU/g instant coffee.

2.5(c) Enzymes Residues in the Final Food

In principle, the hydrolysis of mannans, with the help of mannanase, can be used in the processing of all fruit and vegetable based foods and food ingredients which naturally contain mannans. In these processes, the mannanase is used as a processing aid in food manufacturing and is not added directly to final foodstuffs.

Mannanase from *Aspergillus niger* is used in coffee processing prior to the pasteurization process. The inactivation conditions for mannanase are supported by the pasteurization step. This step requires temperatures held at >90°C for specific periods of time, which is sufficient to inactivate the mannanase.

Furthermore, under a lack of substrate and favourable pH conditions, the enzyme will not be active in the final food application.

Consequently, it can be concluded that the β -mannanase does not exert any enzymatic activity in the final foods to do the conditions indicated above.

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

The assumptions are highly exaggerated since the enzyme protein and the other substances are diluted or removed in certain processing steps. Furthermore, all processed foods and beverages produced with the enzyme are not always produced with the maximum recommended dosage. Therefore, the safety margin calculation derived from this method is highly conservative.

Overall, the human exposure to the mannanase will be negligible because the enzyme preparation is used as a processing aid and in very low dosages therefore the safety margin calculation derived from this method is highly conservative.

3(b) Food Consumption Data

The exposure assessment for adults was performed according to the Budget Method (16) (17) (18). The Budget Method assumptions represent a "maximum worst case" scenario of human consumption. The food enzyme, object of the present dossier, would be used at its maximum recommended dosages in the production of all processed beverages. It is also assumed that the totality of the food enzyme will end up in the final food.

Assumptions in the Budget Method

Liquids: The maximum intake of liquids (other than milk) is 100 ml/kg body weight per day. Assuming, 25% of the non-milk beverages are processed, the daily consumption will be 25 ml processed beverages per kg body weight.

It is further assumed that all processed beverages are liquid coffee.

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

It is assumed that the typical amount of instant coffee used per is 5g per 150ml of water. For the Budget Method, this equates to 0.83g of instant coffee per 25ml water, makes 25ml liquid coffee.

The mannanase has an average activity of 26933 AMNU/g. And approximately 12% TOS (Total Organic Solids) content.

This corresponds to an activity/TOS ratio of 224 AMNU/mg TOS.

The maximum recommended dosage is: 150 AMNU/g of dry instant coffee

This results in a Total Maximum Daily Intake (TMDI) of TOS:

$$0.67 \times 0.83\text{g} = 0.56\text{mg TOS/kg bw/day}$$

Theoretical Maximum Daily Intake (TMDI)

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

The NOAEL dose level in the 13-week oral rat feeding studies conducted on mannanase tox batch PPE42634 was the highest dosage possible, 1152 mg TOS/kg body weight per day. See Table 3 below.

Table 3. NOAEL Calculation

NOAEL (mg TOS/kg bw/day)	1152
*TMDI (mg TOS/kg bw/day)	0.56
Safety margin	2057

*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 determination of general recognition of safety of the mannanase enzyme preparation. Our safety evaluation in Part 6 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) 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). The production organism for the mannanase, *Aspergillus niger*, is discussed in Part 2 and in this Part.

If the organism is non-toxicogenic and non-pathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, is safe to consume (4). Pariza and Foster define a non-toxicogenic 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 is generally considered to be non-pathogenic and non-toxicogenic and is often mentioned as an example of a well characterized and safe production strain with a long history of safe use. *Aspergillus niger* meets the US Environmental Protection Agency (EPA) criteria for non-toxicogenicity and non-pathogenicity, it is one of 10 recipient organisms eligible for Tier I exemption under the EPA regulations (19) and was exempted from EPA review under TSCA (19). Also, the FDA has previously affirmed as GRAS several enzyme preparations from *Aspergillus niger* (GRAS notifications 000089, 000111, 000132) to name a few (20).

An essential aspect of the safety evaluation of food components derived from genetically modified organisms is the identification and characterization of the inserted genetic material (4) (5) (1) (6) (7) (8). The methods used to develop the genetically modified production organism and the specific genetic modifications introduced into the production organism are described in Part 2.

The *Aspergillus niger* production strain is derived from a safe strain lineage comprising production strains for more than ten enzyme preparations which have full toxicological safety studies (i.e. 13-week oral toxicity study in rats, Ames test and/or *In vitro* Micronucleus Test and NRU). See Table 4 below.

Table 4: Safe Strain Lineage

Enzyme	EC No.	Predecessor strain (a)	Donor strain	Safety studies (b)
Glucoamylase	3.2.1.3	<i>Aspergillus niger</i> BO-1	None	Yes
Pectin lyase	4.2.2.10	<i>Aspergillus niger</i> BO-1	<i>Aspergillus niger</i>	Yes
Lysophospholipase	3.1.1.5	<i>Aspergillus niger</i> BO-1	<i>Aspergillus niger</i>	Yes
Triacylglycerol lipase	3.1.1.3	<i>Aspergillus niger</i> BO-1	<i>Candida antarctica</i>	Yes
Glucoamylase	3.2.1.3	<i>Aspergillus niger</i> JaL303	<i>Aspergillus niger</i>	Yes
Glucoamylase	3.2.1.3	<i>Aspergillus niger</i> JaL303	<i>Talaromyces emersonii</i>	Yes
Glucoamylase	3.2.1.3	<i>Aspergillus niger</i> C878	<i>Trametes cingulata</i>	Yes
Alpha-amylase	3.2.1.1	<i>Aspergillus niger</i> C878	<i>Rhizomucor pusillus</i>	Yes
Alpha-amylase	3.2.1.1	<i>Aspergillus niger</i> C2218	<i>Rhizomucor pusillus</i>	Yes
Glucoamylase	3.2.1.3	<i>Aspergillus niger</i> C2218	<i>Gloeophyllum trabeum</i>	Yes
Glucoamylase	3.2.1.3	<i>Aspergillus niger</i> C2218	<i>Penicillium oxalicum</i>	Yes
Triacylglycerol lipase	3.1.1.3	<i>Aspergillus niger</i> C2218	<i>Candida antarctica</i>	Yes
Phospholipase A	3.1.1.32	<i>Aspergillus niger</i> C2948	<i>Talaromyces leycettanus</i>	Yes

Table 4. Novozymes products derived from *A. niger* strains where safety studies have been carried out.

^{a)} The predecessor strain shows strains in the GM construction that are in common with the C40 strain lineage.

^{b)} The following tox studies were performed: *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

Aspergillus niger has a long history of safe use in the production of industrial enzymes and chemicals for use in food (21). *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) (20). 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 158, 183, 214, 296, 345, 402, 428 which all describe food enzymes produced by *Aspergillus niger* strains (20).

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 (19). Also, *Aspergillus niger* was reviewed and was

concluded to be a safe source organism by Olempska-Beer et al. (22) and Schuster et al. (21) under Good Manufacturing Practice (GMP) and with mycotoxin testing.

An evaluation of the genetically modified production microorganism for the mannanase, 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 (16), FAO/WHO in 1996 (7), JECFA in 1998 (13) and Pariza and Johnson in 2001 (3) and more recently outlined in Sewalt et.al (23) demonstrates the safety of this genetically modified production microorganism strain. The components of this evaluation: the identity of the recipient strain, a description of the incorporated DNA, the sources and functions of the introduced genetic material, an outline of the genetic construction of the production strain, and some characteristics of the production strain and the enzyme derived from it are given in Part 2.

Novozymes' used the decision tree (Appendix 1) in Pariza and Johnson 2001 (3) as a basis for our safety assessment. The production strain is genetically modified as discussed in Part 2. The expressed enzyme product is mannanase. The enzyme preparation is free of DNA encoding transferable antibiotic resistant DNA genes. 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 genetic modifications are well characterized and specific utilizing well-known plasmids for the vector constructs, and the introduced genetic material does not encode and express any known harmful or toxic substances.

Some *Aspergillus niger* strains can produce ochratoxin A (21), and the production of fumonisin B2 has also been shown in *Aspergillus niger* (24). 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 (25).

The BO-1 safe strain lineage was found unable to produce unwanted secondary metabolites (ochratoxin A and fumonisin B2) under conditions that are known to induce mycotoxin production in fungi. In addition, analytical test results of three representative batches of this mannanase (as discussed in Part 2.4(b)) confirm the absence of ochratoxin A and fumonisin B2.

Based on critical review and evaluation of its published and unpublished information, Novozymes concludes through scientific procedures that the subject of this notification, mannanase enzyme preparation, meets the appropriate food grade specifications and is produced in accordance with current good manufacturing practices, thus making it GRAS for the intended conditions of use.

6(b) Safety of the Donor Organism

The donor organism of the mannanase is *Talaromyces leycettanus*. As indicated in Part 2, the introduced DNA is well defined and characterized. Only well characterized DNA fragments, limited solely to the mannanase coding sequence from the donor strain, are used in the construction of the genetically modified strain. The introduced DNA does not code for any known harmful or toxic substances.

6(c) Safety of the Mannanase Enzyme

As indicated in Part 2, the subject of this GRAS notification is a mannanase, EC 3.2.1.78. A wide variety of enzymes are used in food processing (2) (3). Enzyme proteins do not generally raise safety concerns (3) (2). Major enzymes such as the B-mannanases (EC 3.2.1.78) are involved in the hydrolysis of linear mannans by hydrolyzing the internal glycosidic bonds of the mannan backbone chain releasing short B-1-4-manno-oligosaccharides (26)

Mannanases are ubiquitous in nature and are commonly found in natural environments. The most mannolytic group among fungi belong to the extracellular genera *Aspergillus*, *Agaricus*, *Trichoderma* and *Sclerotium*. Microbial mannanase are mainly extracellular and can act in a wide range of pH and temperatures (26). *Aspergillus niger* is one of several known sources of mannanases.

Extracellular mannanases are of considerable commercial importance as industrial production of mannans is efficient and easy. Mannanase preparations are used for the hydrolysis of coffee mannan and reduces the viscosity of coffee extracts, positively affecting the processing of instant coffee (27).

As mentioned in Part 2.5(a), mannans are considered a main constituent of hemicellulose. In the Food Standards Australia New Zealand Food (FSANZ) Code (28) – Standard 1.3.3 – processing aids; EC 3.2.1.78 “Hemicellulase multicomponent enzyme” from *Aspergillus niger*, *Bacillus amyloliquefaciens*, *Bacillus subtilis*, and *Trichoderma reesei* are listed as safe for use in food.

According to Pariza and Johnson (3) β -mannanase from *A. niger* has a history of use in food (2). Also, mannanase is the subject of GRAS notice 566, which is included in FDA’s inventory of GRAS substances (20). FDA had no questions concerning the conclusion that mannanase from *T. reesei* is generally recognized as safe for use as a processing aid in coffee production (along with other applications).

A literature search was performed in September 2017 utilizing Medline, PubMed, Scopus, ToxFile, FOODLINE: Science and FSTA (Food Science and Technology Abstracts) using search terms “mannanase” in combination with the terms “toxicology” or “safety” or “human consumption”, resulting in 135 hits. Novozymes

reviewed the available abstracts and found none to be inconsistent with our conclusion of the general recognition of safety for the mannanase enzyme.

Also, the safety of the mannanase enzyme was assessed using the Pariza and Johnson, (2001) decision tree which is included in this submission.

6(d) Allergenic/Toxicogenic Potential of the Mannanase Enzyme

The ingestion of a food enzyme protein is not considered a concern for food allergy. 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 parts per million. 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) (29).

To further evaluate the possibility that the mannanase will cross-react with known allergens and induce a reaction in an already sensitized individual, a sequence homology to known food allergens was assessed. Following the guidelines developed by FAO/WHO, 2001 (30) and modified by Codex Alimentarius Commission, 2009 (31) the mannanase 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 showed no matches. Alignment of the mannanase 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 mannanase and any of the allergens from the databases

mentioned above. And, a search for 100% identity over 8 contiguous amino acids was completed. Again, no homology was found.

Also, a search for homology of the mannanase sequence from *Talaromyces leycettanus* to known toxins was assessed based on the information present in the UNIPROT database (05-03-17). This database contains entries from SWISSPROT and TREMBL. The homology among the emerging entries was below 17%, indicating that the homology to any toxin sequence in this database is low and random.

Consequently, oral intake of the mannanase is not anticipated to pose any food allergenic or toxin concerns.

6(e) Safety of the Manufacturing Process

This section describes the manufacturing process for the mannanase which follows standard industry practices (11) (10) (9). The quality management system used in the manufacturing process for the mannanase 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(f) Safety Studies

This section describes the studies and analysis performed to evaluate the safety of use of the mannanase enzyme.

The following studies were performed on enzyme concentrate test batch PPE42634 with favourable results:

- Reverse Mutation Assay (Ames test)
- *In vitro* Cytogenicity Micronucleus Test in Cultured Human Lymphocytes
- *In vitro* Cytotoxicity Test: Neutral Red Uptake
- 13-week sub-chronic oral toxicity study

These tests are described in Appendix 2. Based on the presented toxicity data and the history of safe use for the strain it can be concluded that mannanase represented by batch PPE42634, exhibits no toxicological effects under the experimental conditions described.

6(g) Results and Conclusion

Results of the toxicity and mutagenicity tests described in Appendix 2 showed no toxicity or mutagenicity of the mannanase from *Aspergillus niger*, PPE42634. A critical review and evaluation of the mannanase from *Aspergillus niger* was done following the concepts of the Pariza papers (2) (3) and the recently described process for the evaluation of GRAS for industrial microbial enzymes by Sewalt et al. (23). This, along with a known history of safe use of the production organism and the limited and well defined nature of the genetic modifications, Novozymes concludes through scientific procedures that the subject of this notification; mannanase enzyme preparation is generally recognized, among qualified experts, to be safe under the conditions of its intended use.

Part 7 – SUPPORTING DATA AND INFORMATION

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

APPENDICES

1. Pariza and Johnson Decision Tree Analysis
2. Summary of Toxicity Data, Mannanase, batch PPE42634. 19 September 2017, LUNA No. 2017-07203-02.
3. Sewalt Vincent, Shanahan Diane, Gregg Lori, La Marta James and Carrillo Roberts; The Generally Recognized as Safe (GRAS) Process for Industrial Microbial Enzymes. *Industrial Biotechnology*, Vol. 12, No. 5. October 2016.

REFERENCES

1. Organisation for Economic Cooperation and Development, Safety Evaluation of Foods Derived by Modern Biotechnology, 1993.
2. Pariza, M.W. and Foster, E.M.. Determining the Safety of Enzymes Used in Food Processing. *Journal of Food Protection*, 46:5:453-468, 1983.
3. Pariza, M.W. and Johnson, E.A.. Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century. *Reg. Tox and Pharm* 33: 173-186, 2001.
4. IFBC (International Food Biotechnology Council). Chapter 4: Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms in Biotechnologies and Food: Assuring the Safety of Foods Produced by Genetic Modification. 1990, Vol. 12, pp. S1-S196.
5. EU Scientific Committee for Food. Guidelines for the presentation of data on food enzymes. Reports of the Scientific Committee for Food, 27th series, 1991.
6. Berkowitz, D. and Maryanski, J.. Implications of biotechnology on international food standards and codes of practice. Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission, Eighteenth Session, Geneva, July 3-12, 1989.
7. FAO/WHO. Biotechnology and Food Safety, Report of a Joint FAO/WHO Consultation. FAO Food and Nutrition Paper 61. Rome, Italy. 1996.
8. Jonas, D.A., et al. The Safety Assessment of Novel Foods, Guidelines prepared by ILSI Europe Novel Food Task Force, *Food Chemical Toxicology*, Vol. 34, 1996. pp. 931-940.
9. Aunstrup, K., Andersen, O., Falch, E.A., and Nielsen, T.K.. Production of Microbial Enzymes in *Microbial Technology*, 2nd ed., Vol. I, Eds. Peppler, H.J. and Perlman, D., Chapter 9, pp.282-309, 1979.
10. Aunstrup, K.. Production, Isolation, and Economics of Extracellular Enzymes in *Applied Biochemistry and Bioengineering*, Volume 2, Enzyme Technology, Eds. Wingard, L.B., Katchalski-Katzir, E. And Goldstein, L, pp. 28-68, 1979.
11. Kirk, O., Damhus, T., Borchert, T. V., Fuglsang, C. C., Olsen, H. S., Hansen, T. T., et al. Enzyme Applications, Industrial. Kirk-Othmer Encyclopedia of Chemical Technology, Vol. 9, pp 566-620, 2000.

12. United States Pharmacopeial Convention. Food Chemical Codex. Edition 10. Monograph: Enzyme Preparations. United States Pharmacopeial Convention, Board of Trustees, 2016. Pg 445-450.
13. JECFA (Joint FAO/WHO Expert Committee on Food Additives. General Specifications and Considerations for Enzyme Preparations Used in Food Processing. Compendium of Food Additive Specifications, FAO FNP (Food and Nutrition Paper) 52, Add. 9, FAO, Rome 2001 and FAO JECFA Monographs 3 (2006).
14. Moreira LR, Filho EX. An overview of mannan structure and mannan-degrading enzyme systems. *Appl Microbiol Biotechnol*. May;79(2):165-78, 2008.
15. Alois Sachslehner, Gabriele Foidl, Nikolaus Foidl, Georg Gubitz, Dietmar Haltrich; Hydrolysis of isolated coffee mannan and coffee extract by mannanases of *Sclerotium rolfsii*. *Journal of Biotechnology*, 80, 127-134, 2000.
16. ILSI Europe Food Chemical Intake Task Force. An Evaluation of the Budget Method for Screening Food Additive Intake. April 1997.
17. Douglass J.S., Barray L.M., Tennant D.R., Long W.R. and Chaisson, C.F.. Evaluation of the budget method for screening food additive intakes, *Food Add. Contam.*, 14, 791-802, 1997.
18. Hansen S.C. (1966). Acceptable daily intake of food additives and ceiling on levels of use, *Food Cosmet. Toxicol*. 4: 427-432, 1966. .
19. US Environmental Protection Agency. Final risk assessment and decision document to exempt *A. niger* strains from the TSCA Biotechnology Program, 1997.
20. GRAS Notice Inventory: <http://www.accessdata.fda.gov>.
21. Schuster, E.; Dunn-Coleman, N.; Frisvad, J.C.; van Dijck, P.W.M. On the safety of *Aspergillus niger* - a review. *Appl. Microbiol. biotechnol*. 59, 426-435. 2002.
22. Olempska-Beer, Z.S. , Merker, R.I, Ditto, Mary D., and DiNovi, M.J. Food-processing enzymes from recombinant microorganisms—a review. *Reg. Tox and Pharm* 45:144-158, 2006.
23. Sewalt Vincent, Shanahan Diane, Gregg Lori, La Marta James and Carrillo Roberts; The Generally Recognized as Safe (GRAS) Process for Industrial Microbial Enzymes. *Industrial Biotechnology*, Vol. 12, No. 5. October 2016.
24. Frisvad JC, Smedsgaard J, Samson RA, Larsen TO, Thrane U. Fumonisin B2 Production by *Aspergillus niger*. *J. Agric. Food Chem*. 55, 9727–9732. 2007.

25. Nielsen KF, Mogensen JM, Johansen M, Larsen TO, Frisvad JC. Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. *Anal Bioanal Chem*, 395, 1225–1242. 2009.
26. Prakram Singh Chauhan, Neena Puri, Prince Sharma and Naveen Gupta. Mannanases: microbial sources, production, properties and potential biotechnological applications. *Appl. Microbiol Biotechnol*, 93:1817-1830, 2012.
27. Dhawan and Kaur Jagdeep; Microbial Mannanases: An Overview of Production and Applications. *Critical Reviews in Biotechnology*, 27:197-216, 2007.
28. Australia New Zealand Food Standards Code (FSANZ). Assessment Report: Explanatory Statement, Proposal P276. review of Processing Aids (Enzymes). February 15, 2007.
29. Bindsvlev-Jensen C, Skov PS, Roggen EL, Hvass P, Brinch DS Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry. *Food Chem. Toxicol.* 44, 1909-1915, 2006.
30. Evaluation of Allergenicity of Genetically Modified Foods (Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology 22-25 January 2001), Food and Agriculture Organization of the United Nations (FAO), Rome, 2001.
31. Codex Alimentarius commission. Foods derived from modern biotechnology. FAO/WHO, Rome, pp.1-85, 2009.

Appendix 3- This mannanase enzyme preparation from *Talaromyces leycettanus* produced by *Aspergillus niger* was evaluated according to the decision tree published in Pariza and Johnson, 2001 ⁽¹⁾.

The result of the evaluation is presented below.

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, go to 3c.

 - c. Is the test article free of transferable antibiotic resistance gene DNA?
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, go to 4.

4. Is the introduced DNA randomly integrated into the chromosome?
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. *If yes the test article is ACCEPTED.*

LIST OF REFERENCES

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



Toxicology & Product Safety

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SUMMARY OF TOXICITY DATA

Mannanase, batch PPE42634, from *Aspergillus niger*

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1. ABSTRACT

The below series of toxicological studies were undertaken to evaluate the safety of Mannanase, batch PPE42634.

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) and Covance (UK) during the period December 2016 to April 2017.

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

- Mannanase, batch PPE42634, was tested in a Neutral Red Uptake assay applying the BALB/c 3T3 cell line as test system and the IC₅₀ was determined to be above 30 mg mg/mL since the relative cytotoxicity for the test substance did not fall below 86 % under the conditions of the assay.
- Mannanase, batch PPE42634, did not induce gene mutations in the Ames test, in the absence or presence of a rat liver metabolic activation system (S-9).
- Mannanase, batch PPE42634, 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).
- In a 13-week oral toxicity study in rats Mannanase, batch PPE42634 was well tolerated and did not cause any toxicologically significant changes at any dose level tested.

Based on the present toxicity data it can be concluded that Mannanase, represented by batch PPE42634, exhibits no significant toxicological effects under the experimental conditions described.

2. TEST SUBSTANCE

The test substance is a mannanase (E.C. 3.2.1.78).

2.1 Characterization

The toxbatch Mannanase, batch PPE42634, 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 Mannanase, batch PPE42634

Batch number	PPE42634
Activity	16700 AMNU/g
N-Total (% w/w)	0.96
Water (KF) (% w/w)	87.8
Dry matter (% w/w)	12.2
Ash (% w/w)	1.2
Total Organic Solids (TOS ¹) (% w/w)	11.0
Specific gravity (g/mL)	1.047

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

3. MUTAGENICITY

3.1 Bacterial Reverse Mutation assay (Ames test)

Mannanase, batch PPE42634 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 the test article may contain free amino acids, i.e. histidine and tryptophan (which may cause artefacts through growth stimulation in a standard plate-incorporation test).

All Mannanase, batch PPE42634 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 11.0% w/w, using a correction factor of 9.09.

Experiment 1 treatments of all the tester strains were performed in the absence and in the presence of S-9, using final concentrations of Mannanase, batch PPE42634 at 16, 50, 160, 500, 1600 and 5000 µg TOS/mL, plus vehicle and positive controls. Following these treatments, no clear evidence of toxicity was observed.

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 Mannanase, batch PPE42634 approaching the maximum test concentration and therefore considered most likely to provide evidence of any mutagenic activity. Following these treatments, no clear evidence of toxicity was observed.

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 Mannanase, batch PPE42634 treatments of all the test strains in the absence and presence of S-9, no increases in revertant numbers were observed that were ≥ 2 -fold (in strains TA98, TA100 and WP2 *uvrA* pKM101) or ≥ 3 -fold (in strains TA1535 and TA1537) the concurrent vehicle control. Small but still notable increases (maximum of 2.3 or 2.5-fold increases, which therefore fell below the 3-fold threshold) were observed following Experiment 2 treatments of strains TA1535 and TA1537 in the absence of S-9. These increases neither achieved the 3-fold threshold, nor were clearly concentration-related or reproducible, and therefore this study was considered to have provided no clear evidence of any Mannanase, batch PPE 42634 mutagenic activity in this assay system.

It was concluded that Mannanase, batch PPE42634 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

Mannanase, batch PPE42634 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 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 Mannanase, batch PPE42634 on the replication index (RI). Micronuclei were tested up to 5000 µg TOS/mL.

Appropriate negative (vehicle) control cultures were included in the test system under each treatment condition. The proportion of micronucleated binucleate (MNBN) cells in the 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 Mannanase, batch PPE42634 in the absence and presence of 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 all concentrations analysed (all treatments). With the exception of one single treated culture (replicate 'A' at a concentration of 3000 µg TOS/mL, following 24+24 hour-S-9 treatment) the MNBN cell frequency of all Mannanase, batch PPE42634 treated cultures (all concentrations) fell within the 95th percentile of the current observed historical vehicle control (normal) ranges.

It is concluded that Mannanase, batch PPE42634 did not induce 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 in accordance with current regulatory guidelines for the *in vitro* micronucleus assay.

4. GENERAL TOXICITY

4.1 *In Vitro* Cytotoxicity Test: Neutral Red Uptake in BALB/c 3T3 Cell Culture

The aim of this study was to evaluate the cytotoxicity of Mannanase, batch PPE42634, using a Neutral Red Uptake (NRU) assay in 3T3 cells.

The growth of 3T3 cells treated with a range of concentrations of the test item was compared with vehicle control cultures after 48 hours exposure both visually and using neutral red uptake.

The relative toxicity for Mannanase did not fall below 86% of the vehicle control at any concentration and was 95% at the highest concentration, 30 mg/mL, indicating that Mannanase had shown no evidence of causing cytotoxicity. Visual assessment of the cell monolayers at the end of the 48 hour treatment indicated that the cells were approximately 70% confluent at all concentrations of Mannanase and the vehicle control.

The IC50 value of the positive control, sodium lauryl sulphate, was calculated to be 95.69 µg/mL, which lay within the historical control range of this laboratory.

It was concluded that Mannanase demonstrated no evidence of causing cytotoxicity in this assay.

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

The purpose of this study was to assess the systemic toxic potential of Mannanase, batch PPE42634 (an enzyme used in the food industry), when administered orally by gavage to Han Wistar rats for 13 weeks. Three groups, each comprising ten males and ten females, received doses of 10, 33 or 100% of Mannanase, batch PPE42634 (equivalent to 115.17, 380.06 and 1151.7 mg TOS/kg body weight/day, or 17484.9, 57700.17 or 174849 AMNU/kg body weight/day). A similarly constituted control group received the vehicle (reverse osmosis water) at the same volume-dose (10 mL/kg body weight) as the treated groups.

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

There was no death and no clinical sign or behavioral finding that were attributable to treatment. In addition there was no effect of treatment on bodyweight or food and water consumption.

There was no treatment-related ophthalmoscopic finding.

The haematological analysis of the peripheral blood and biochemical analysis of the plasma in Week 13 did not identify any difference from controls that was attributable to treatment.

Organ weights were unaffected after 13 weeks and there was no treatment related macroscopic or histopathological finding.

It is concluded that oral administration of Mannanase, batch PPE42634 to Han Wistar rats at doses up to 100% of the Mannanase (equivalent to 1151.7 mg TOS/kg body weight/day or 174849 AMNU/kg body weight/day) for 13 weeks was well tolerated and there was no significant finding of any toxicological relevance. The no-observed-adverse-effect-level (NOAEL) in this study was considered to be 100% Mannanase, batch PPE42634 (equivalent to 1151.7 mg TOS/kg body weight/day or 174849 AMNU/kg body weight/day).

5. REFERENCES

5.1 Study reports

Envigo: Study No.: WM16DN; Novozymes Reference No.: 20166072: Mannanase, Batch PPE42634: 3T3 Neutral Red Uptake test. (January 2017). LUNA file: 2017-01475.

Covance: Study No.: 8356289; Novozymes Reference No.: 20166076. Mannanase, Batch PPE42634: Bacterial Reverse Mutation Assay using a Treat and Plate Modification. (April 2017). LUNA file: 2017-06684.

Covance: Study No.: 8356290; Novozymes Reference No.: 20166077: Mannanase, Batch PPE42634: *In vitro* Human Lymphocyte Micronucleus Assay. (March 2017). LUNA file: 2017-04288.

Envigo: Study No.: NT71RB; Novozymes Reference No.: 20166061: Mannanase, Batch PPE42634: Toxicity Study by Oral Gavage Administration to Han Wistar Rats for 13 Weeks. (September 2017). LUNA file: 2017-13903.