ORIGINAL SUBMISSION

Form Approved: OMB No. 0910-0342; Expiration Date: 02/29/2016
(See last page for OMB Statement)

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
000680	11/16/2016
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 Specialist III

Company *(if applicable)*: Novozymes

Mailing Address *(number and street)*: 77 Perry 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
919-494-3187		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

000002

PART III – GENERAL ADMINISTRATIVE INFORMATION

1. Name of Substance

alpha-L-arabinofuranosidase from Talaromyces pinophilus produced by Trichoderma reesei

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).*

This alpha-L-arabinofuranosidase enzyme preparation is intended for use as a processing aid to increase the yield of starch and gluten meal during the corn wet milling process by contributing to the degradation of the plant's cell wall matrix. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following cGMP. The dosage applied in practice by a food manufacturer depends on the particular process. It is based on an initial recommendation by the enzyme manufacturer and optimized to fit the process conditions.
The maximum recommended use level is 90 ARXU(M) per kilo of corn dry matter.

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

000003

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-L-arabinofuranosidase	EC	3.2.1.55		
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-L-arabinofuranosidases are produced by bacteria and fungi. Alpha-L-arabinofuranosidases are glycoside hydrolases that specifically hydrolyze non-reducing residues from arabinose-containing polysaccharides. In the case of arabinoxylans, which are the main components of hemicellulose, they are part of microbial xylanolytic systems and are necessary for complete breakdown of arabinoxylans

3. Synonyms

Provide as available or relevant:

1	non-reducing end α-L-arabinofuranosidase
2	
3	

000004

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 *(name of notifier)*

has concluded that the intended use*(s)* of alpha-L-arabinofuranosidase from Talaromyces pinophilus produced by Trichoderma reesei *(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 *(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 *(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 Perry Chapel Church Rd, Franklinton, NC 27525 *(address of notifier or other location)*

Novozymes *(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: 2016.11.14 11:17:01 -05'00'	Janet Oesterling, Regulatory Specialist III	11/14/2016

000005

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-L-arabinofuranosidase from T. reesei	Submission
	DecisionTree_Appendix 2_alpha-L-arabinofuranosidase from T. reesei	Administrative
	SafetyofMicrobialEnzymePreps_ParizaandJohnson_April2001	Administrative
	SummaryofToxicityData_alpha-L-arabinofuranosidase_2016-11-14	Administrative
	Part 1_alpha-L-arabinofuranosidase from T. reesei_2016-11-14	Administrative

OMB Statement: Public reporting burden for this collection of information is estimated to average 150 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.

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 Alpha-L-arabinofuranosidase from *Talaromyces pinophilus* produced by *Trichoderma reesei.*

§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:

This alpha-L-arabinofuranosidase enzyme preparation is intended for use as a processing aid to increase the yield of starch and gluten meal during the corn wet milling process by contributing to the degradation of the plant's cell wall matrix. 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)

11-14-16

Janet Oesterling Date
Regulatory Affairs Specialist III

Alpha-L-arabinofuranosidase from *Talaromyces pinophilus* produced by *Trichoderma reesei*

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

November 2016

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-L-arabinofuranosidase enzyme preparation produced by submerged fermentation of a genetically modified *Trichoderma reesei* microorganism carrying the gene coding for alpha-L-arabinofuranosidase from *Talaromyces pinophilus.*

Key enzyme and protein chemical characteristics of the alpha-L-arabinofuranosidase are given below:

Classification:	alpha-L-arabinofuranosidase
IUBMB nomenclature:	non-reducing end α-L-arabinofuranosidase
EC No.:	3.2.1.55
CAS No.:	9067-74-7
Specificity:	α-L-arabinofuranosidases specifically cleave either an α-1,2 or α-1,3-L-arabinofuranose side chains from arabinoxylan.
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 *Trichoderma reesei (T. reesei)* production strain, designated AmFs272-31-b1, was derived from recipient strain BTR213, a natural isolate of *T. reesei* strain RUTC30 (ATCC 56765) (1). RUTC30 is derived from the well-known wild type strain QM6a. QM6a is the parent of practically all *T. reesei* industrial production strains (2). *T. reesei* is classified as a Biosafety Level 1 microorganism by the American Type Culture Collection (ATCC) based on risk assessment from U.S. department of Public Health guidelines (3).

T. reesei complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms (4). It also meets the criteria for a safe production microorganism as described by Pariza and Foster (5) and later Pariza and Johnson (6) and several expert groups (7) (2) (8) (9).

The expression plasmid, used in the strain construction, *pAmFs272*, contains strictly defined chromosomal DNA fragments and synthetic DNA linker sequences. The DNA sequence for the introduced gene is based on the *afuTP* sequence encoding an alpha-L-arabinofuranosidase from *Talaromyces pinophilus*.

2.2(b) Recipient Strain

The recipient strain BTR213 used in the construction of the alpha-L-arabinofuranosidase production strain (AmFs272-31-b1) was modified by several rounds of classical mutagenesis of RUTC30, screening for increased level of enzyme production.

2.2(c) Alpha-L-arabinofuranosidase Expression Plasmid

The expression plasmid, pAmFs272, used to introduce the *afuTP* gene in the recipient strain BTR213 is based on the replication origin of *E. coli*. However, no fragments of the vector backbone are introduced into the production strain. The plasmid contains the expression cassette consisting of a fragment of the *T. reesei cbh1* (cellobiohydrolase 1) promotor, the *afuTP* gene encoding the alpha-L-arabinofuranosidase, the transcriptional terminator of *cbh1* and a 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 analysis and PCR analysis followed by DNA sequencing.

2.2(d) Construction of the Recombinant Microorganism

The production strain, *Trichoderma reesei* AmFs272-31-b1, was constructed from the recipient strain BTR213 through the following steps:

1. The expression cassette from plasmid pAmFs272 was integrated into one specific locus in strain BTR213 by targeted homologous recombination to this locus. Targeted integration allows the expression of the *afuTP* gene from the promoter.

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

The resulting alpha-L-arabinofuranosidase production strain containing one copy of the *afuTP* gene at the target locus was named AmFs272-31-b1.

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

2.1(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 end of production using an alpha-L-arabinofuranosidase gene specific probe showed an identical band pattern compared to the reference production strain (AmFs272-31-b1), demonstrating the genetic stability of the introduced DNA during production. The transforming DNA is stably

integrated into the *T. reesei* 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 (10) is satisfactorily addressed.

2.3 METHOD OF MANUFACTURE

This section of Part 2 describes the manufacturing process for the enzyme which follows standard industry practices (11) (12) (13). The quality management system used in the manufacturing process for the alpha-L-arabinofuranosidase complies with the requirements of ISO 9001. It is produced under a standard manufacturing process as outlined by Aunstrup (12) and 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 (14). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (15).

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 (11) (12) (13). 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 alpha-L-arabinofuranosidase enzyme preparation is produced by pure culture submerged fed-batch fermentation of a genetically modified strain of *T. reesei* as described in Part 2. All equipment is carefully designed, constructed, operated, cleaned, and maintained so as 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, *T. reesei,* 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 standardized with sucrose. The liquid product is formulated by 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 alpha-L-arabinofuranosidase 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*)	11%
Water	45 - 65%
Sucrose	20 - 35%
Sodium Benzoate	<0.5%
Potassium Sorbate	<0.5%

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

2.4(b) Specifications

The alpha-L-Arabinofuranosidase enzyme preparation complies with the recommended purity specification criteria for “Enzyme Preparations” as described in *Food Chemicals Codex* (14). 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 (15).

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

Table 2. Analytical data for three food enzyme batches

Parameter	Specification	PPH40203	PPH40223	PPH40645
Activity unit	ARXU(M)/g	313	297	322
Lead	Not more than 5 mg/kg	<0.5	<0.5	<0.5
Total Coliforms	Not more than 30/g	4	<4	<4
Salmonella	Absent in 25g	ND	ND	ND
Escherichia coli	Absent in 25g	ND	ND	ND
Antimicrobial activity	Not detected	ND	ND	ND

2.5 PHYSICAL OR TECHNICAL EFFECT

2.5(a) Mode of Action

The active enzyme is an alpha-L-arabinofuranosidase (EC 3.2.1.55). Alpha-L-arabinofuranosidase specifically cleave either an α-1, 2 or α-1, 3-L-arabinofuranose side chains from arabinoxylan. Arabinoxylans are highly branched xylans found in the outer cell walls and endosperm of cereal grains such as corn, wheat, barley, rye and oat. The alpha-L-arabinofuranosidase preparation is used during food processing to aid in the separation of grains into the germ, starch, gluten and fibers.

2.5(b) Use Levels

The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following cGMP.

The dosage applied in practice by a food manufacturer depends on the particular 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 90 ARXU(M) per kilo of corn dry matter.

2.5(c) Enzymes Residues in the Final Food

The alpha-L-arabinofuranosidase enzyme is to be used in the wet milling process for separation of corn into germ, starch, gluten and fibers. The enzyme treatment of the foodstuffs is taking place upstream and the enzyme is heat inactivated during subsequent heat treatment steps, i.e. in the drying steps for fiber and gluten or in the further processing of the starch product to i.e. syrup.

PART 3 - DIETARY EXPOSURE

The alpha-L-arabinofuranosidase enzyme is to be used in the wet milling process for separation of corn into germ, starch, gluten and fibers. The enzyme treatment is taking place upstream and the enzyme is largely heat inactivated during subsequent heat treatment steps.

However, in order 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.

The alpha-L-arabinofuranosidase has an average activity of 310 ARXU(M)/g and approximately 11% TOS (Total Organic Solids) content.

This corresponds to an activity/TOS ratio of 2.82 ARXU(M)/mg TOS.

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.

The exposure assessment is based on the Budget Method (16) which represents a “maximum worst case” situation of human consumption. Overall, the human exposure to the alpha-L-arabinofuranosidase 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 – Budget Method

The alpha-L-arabinofuranosidase enzyme is to be used in the wet milling process for separation of corn into germ, starch, gluten and fibers. The enzyme treatment of the foodstuffs is taking place upstream and the enzyme is heat inactivated during subsequent heat treatment steps, i.e. in the drying steps for fiber and gluten or in the further processing of the starch product to i.e. syrup.

An exposure assessment of the enzyme preparation, according to the Budget Method, has been performed used as an ingredient that consumers are likely to eat as part of a total diet in a variety of food products.

The calculations are generated on the basis of the maximum amounts of the food enzyme that could theoretically be carried-over to final food and drinks.

Solid Food: The maximum energy intake over the course of a lifetime is 50 kcal/kg body weight (b/w) /day. Fifty 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.

It is further assumed that, on average, all processed food contains 25% starch (or starch-derived) dry matter = 3.12 g starch derived dry matter per kg bw per day.

The maximum recommended dosage is: 90 ARXU(M)/kg dry corn matter.

The starch content in corn is assumed to be 70%. The highest dosage of enzyme activity per kilo of dry corn matter is therefore:

90 ARXU(M)/kg corn dry matter ÷ 0.70 kg starch in corn dry matter = 132 ARXU(M)/kg starch dry matter.

This corresponds to 46.80 mg TOS

Based on this 3.12 gram starch derived dry matter in solid food will maximally contain:

46.80 mg TOS per kg / 1000 g per kg x 3.12 g = 0.146 mg TOS/kg bw/day

Theoretical Maximum Daily Intake (TMDI)

The safety margin is calculated as dose level with no adverse effect (NOAEL) divided by the estimated human consumption. The NOAEL dose level in the 13 weeks oral toxicity study in rats conducted on alpha-L-arabinofuranosidase, PPH40331 was the highest dosage possible, 1116 mg TOS/kg bw/day. See Appendix 3 and Table 3 below.

Table 3. NOAEL Calculation

NOAEL (mg TOS/kg bw/day)	1116
*TMDI (mg TOS/kg bw/day)	0.146
Safety margin	7644

*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 alpha-L-arabinofuranosidase 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.

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 (6) (5) (Appendix 1). The production organism for the alpha-L-arabinofuranosidase, *Trichoderma reesei,* is discussed in Part 2 and also in this Part. The names *Trichoderma reesei, Trichoderma longibrachiatum,* and *Hypocrea jecorina* may appear in different documents, but they refer to essentially the same fungal species.

T. reesei has a long history of safe use in industrial scale enzyme production. The safety of this species as an industrial enzyme producer has been reviewed (2) (8). The organism is considered non-pathogenic for humans and does not produce fungal toxins or antibiotics under conditions used for enzyme production. It is generally considered a safe production organism and is the source organism of a range of enzyme products that are used as processing aids in the international food industries (2) (8).

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 (10) (17) (4) (18) (19) (20). 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.

6(a) Safety of the Production Organism

The safety of the *T. reesei* production organism must be the prime consideration in assessing the degree of safety of an enzyme preparation intended for use in food (21) (6). 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, is safe to consume (22). Pariza and Foster (5) define a non-toxigenic organism as “one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure” and a non-pathogenic organism as “one that is very unlikely to produce disease under ordinary circumstances”.

T. reesei has a long history (more than 35 years) of safe use in industrial scale enzyme production and can be considered as a safe production organism for enzymes for food as well as feed processing and numerous other industrial

applications. The original isolate, QM6a, and its subsequent derivatives have been the subject of intense research due to their usefulness in the production of cellulases. T. reesei is not present on the list of pathogens used by the EU (Directive Council Directive 90/679/EEC) and major culture collections worldwide (23). It is classified as a Biosafety Level 1 (BSL 1) microorganism by the American Type Culture Collection (ATCC) based on assessment of the potential risk using U.S. Department of Public Health guidelines. BSL 1 microorganisms are not known to cause diseases in healthy adult humans.

Cellulases, hemicellulases, beta-glucanases, pectinases and xylanases produced by this fungus are used in food, animal feed, pharmaceutical, textile, detergent, bioethanol and pulp and paper industries (2) (8) (24). Trichoderma reesei strains are non-pathogenic for healthy humans and animals (2). The safety of *T. reesei* has been discussed in several review papers (2) (8) (25) (26). *T. reesei* has been described not to produce mycotoxins or antibiotics under conditions used for enzyme production.

All fungal species produce secondary metabolites to allow them to survive in nature. It is recognized that *T. reesei* is capable of producing a peptaibol compound (paracelsin) (25). However, the bulk of the literature investigating the capability of *T. reesei* to produce peptaibol is based on fermentation conditions designed either to mimic natural (and poor) growth conditions or attempt to optimize the conditions for secondary metabolite production. These methods are not representative of the conditions used in controlled industrial fermentation practices (27) (28) (25).

In 2012, the US EPA published a risk assessment (29) to support tiered exemption status for *T. reesei* QM6a and it's derivate. The EPA acknowledged in this assessment that under normal submerged fermentation conditions paracelsin is not produced. Novozymes has confirmed, by testing, that paracelsin is not produced by the production strain (AmFs272-31-b1).

Enzyme preparations from *T. reesei* have been approved for use in food in Canada (Food and Drugs Act Division 16, Table V), France (Arrêté du 19 Octobre 2006), Denmark, Australia/New Zealand (Standard 1.3.3 processing aids), China, and Japan. To this date, there are ten enzymes produced in T. reesei that have been notified to FDA/CFSAN as GRAS for their intended uses (30). In addition, cellulase enzyme preparation from *T. reesei* is the subject of the regulation in 21 CFR §184.1250.

An evaluation of the genetically modified *T. reesei* production organism embodying the concepts initially outlined by Pariza and Foster, 1983 (21) and further developed by IFBC in 1990 (22), the EU SCF in 1991 (17), the OECD in 1992 (4), ILSI Europe Novel Food Task Force in 1996 (20), FAO/WHO in 1996 (19), JECFA in 1998 (15) and Pariza and Johnson in 2001 (6), 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 2) in Pariza and Johnson 2001 (6) as a basis for our safety assessment. The production strain is genetically modified as discussed in Part 2. The expressed enzyme product, alpha-L-arabinofuranosidase, has a history of safe use in food. The enzyme preparation is free of DNA encoding transferable antibiotic resistance 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.

Based on the information presented here it is concluded that the *T. reesei* production strain is considered a safe strain for the production of alpha-L-arabinofuranosidase enzyme (8) (2).

6(b) Safety of the Donor Organism

The donor organism of the alpha-L-arabinofuranosidase is *Talaromyces pinophilus*. As indicated in Part 2, the introduced DNA is well defined and characterized. Only well characterized DNA fragments, limited solely to the alpha-L-arabinofuranosidase 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 Alpha-L-Arabinofuranosidase Enzyme

As indicated in Part 2, the subject of this GRAS notification is an alpha-L-arabinofuranosidase, EC 3.2.1.55. Enzymes, including alpha-L-arabinofuranosidase, have a long history of use in food (6) (5) and animal feed (31).

Alpha-L-arabinofuranosidases are produced by bacteria (32) and fungi (33). Alpha-L-arabinofuranosidases are glycoside hydrolases that specifically hydrolyze non-reducing residues from arabinose-containing polysaccharides. In the case of arabinoxylans, which are the main components of hemicellulose, they are part of microbial xylanolytic systems and are necessary for complete breakdown of arabinoxylans (34).

The importance of lignocellulose degrading enzymes, such as alpha-L-arabinofuranosidases, is well defined because of their role in many industrial and biotechnological processes. These include production of important medicinal compounds, improvement of wine flavors, bread quality, pulp treatment, juice clarification, quality of animal feedstock, production of bioethanol and the synthesis of oligosaccharides etc. (35) (24).

The active enzyme is alpha-L-arabinofuranosidase. Alpha-L-arabinofuranosidase (EC 3.2.1.55) was mentioned as a systematic name for the positive evaluation of hemicellulases from *A. niger* enzyme preparation, by JECFA (36).

Alpha-L-arabinofuranosidase has been evaluated as an enzyme produced from *A. niger* (37) and is also the subject of extensive research (38) (39) (40) (24) in a variety of industrial and commercial uses.

Enzyme proteins do not generally raise safety concerns (6) (5) . Pariza and Foster (5) note that very few toxic agents have enzymatic properties. The safety of the alpha-L-arabinofuranosidase was assessed using the Pariza and Johnson, (2001) decision tree (Appendix 2).

Based on the information presented above, it is concluded that alpha-L-arabinofuranosidase enzymes have a history of safe use and do not have toxic properties.

6(d) Allergenic/Toxigenic Potential of the Alpha-L-Arabinofuranosidase 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) (41).

In order to further evaluate the possibility that the alpha-L-arabinofuranosidase 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 (42) and modified by Codex Alimentarius Commission, 2009 (43) the alpha-L-arabinofuranosidase 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.

More than 35% identity in the amino acid sequence of the expressed protein using a window of 80 amino acids and a suitable gap penalty showed no matches. Alignment of the alpha-L-arabinofuranosidase to each of the allergens and identity of hits with more than 35% identity over the full length of the alignment was analyzed. No significant homology was found between the alpha-L-arabinofuranosidase 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 significant homology was found.

Also, a sequence homology of alpha-L-arabinofuranosidase produced by strain AmFs272-31-b1 to known toxins was assessed on the basis of the information present in the UNIPROT database (02-Feb-2016). This database contains entries from SWISSPROT and TREMBL. The homology among the emerging entries was below 20% indicating that the homology to any toxin sequence in this database is low and random.

On the basis of the available evidence it is concluded that oral intake of alpha-L-arabinofuranosidase produced by *T. reesei* is not anticipated to pose any food allergenic or toxigenic concerns.

6(e) Safety of the Manufacturing Process

The alpha-L-arabinofuranosidase enzyme preparation meets the purity criteria for enzyme preparations as outlined in the monograph on Enzyme Preparations in the *Food Chemicals Codex.* As described in Section 3, the enzyme preparation is produced in accordance with current good manufacturing practices, using ingredients that are acceptable 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 (13) (11) (12).

6(f) Safety Studies

This section describes the studies and analysis performed to evaluate the safety of the use of the alpha-L-arabinofuranosidase.

The following studies were performed on test batch PPH40331 with favourable results:

- Reverse Mutation Assay (Ames test)
- *In vitro* Micronucleus Test In Cultured Human Lymphocytes

- 13 week sub-chronic oral toxicity study

These tests are described in Appendix 3. Based on the presented toxicity data and the history of safe use for the strain it can be concluded that alpha-L-arabinofuranosidase, represented by batch PPH40331, exhibits no toxicological effects under the experimental conditions described.

6(g) Results and Conclusion

Novozymes has reviewed the available data and information. We are not aware of any data and/or information that is, or appears to be, inconsistent with our conclusion of GRAS. Based on this critical review and evaluation, a history of safe use of *T. reesei* and the limited and well defined nature of the genetic modifications, Novozymes concludes through scientific procedures that the subject of this notification; arabinofuranosidase enzyme preparation, meets the appropriate food grade specifications and is produced in accordance with current good manufacturing practices. Thus, it 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, M.W. and Johnson, E.A. Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century Regulatory, Toxicology and Pharm 33: 173-186, 2001.

2 Pariza and Johnson Decision Tree Analysis

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Appendix 2- Pariza & Johnson Decision Tree analysis of an alpha-L-arabinofuranosidase from *Talaromyces pinophilus* produced by *Trichoderma reesei*

This alpha-L-arabinofuranosidase from *Talaromyces pinophilus* produced by *Trichoderma reesei* 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.

> 3a. Does the expressed enzyme product which is encoded by the introduced DNA have a history of safe use in food?
> **YES, go to 3c**
>
> 3c. Is the test article free of transferable antibiotic resistance gene DNA?
> ***YES, go to 3e.***
>
> 3e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food 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, *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.

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Toxicology & Product Safety

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SUMMARY OF TOXICITY DATA

Arabinofuranosidase, batch PPH40331 from *Trichoderma reesei*

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CONTENTS

PAGE

1. ABSTRACT

The below series of toxicological studies were undertaken to evaluate the safety of Arabinofuranosidase, batch PPH40331.

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 2015 to July 2016.

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

- Arabinofuranosidase, batch PPH40331 was well-tolerated and did not cause any adverse change after oral exposure for 13 weeks. The no-observed adverse-effect level (NOAEL) was considered to be high dose equivalent to 10mL pr kg bw daily for 13-weeks corresponding to 1116 mg TOS/kg/day.

- It was concluded that Arabinofuranosidase, PPH40331 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 in the absence and in the presence of a rat liver metabolic activation system (S-9) using a modified Treat and Plate methodology.

- Arabinofuranosidase, batch PPH40331 did not induce biologically relevant increases in micronuclei in cultured human peripheral blood lymphocytes following treatment in the absence and presence of a rat liver metabolic activation system (S-9). Concentrations were tested up to 5000 µg TOS/mL.

Based on the present toxicity data and the history of safe use for the strain it can be concluded that Arabinofuranosidase, represented by batch PPH40331, exhibits no significant toxicological effects under the experimental conditions described.

2. TEST SUBSTANCE

Arabinofuranosidase (IUBMB/E.C. 3.2.1.55) catalyzes the chemical hydrolysis of terminal non-reducing alpha-L-arabinofuranoside residues in alpha-L-arabinosides. The enzyme is used in the corn industry to increase starch and gluten yields during wet milling.

2.1 Characterization

The batch Arabinofuranosidase, batch PPH40331 was used for the conduct of all the toxicological studies. The characterization data of the batch is presented in Table 1.

Table 1. Characterization data of Arabinofuranosidase, batch PPH40331

Batch number	PPH40331
Activity (ARXU(M)DV/g)	300.3
BCA_Total mg/g	128.9
BCA(B) mg/g	117.4
N-Total (% w/w)	1.52
Water (KF) (% w/w)	88.7
Dry matter (% w/w)	11.3
Ash (% w/w)	0.6
Total Organic Solids (TOS[1]) (% w/w)	10.7

Specific gravity (g/mL)	1.043

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

3. GENERAL TOXICITY

3.1 13-Week Oral Toxicity Study in Rats

The objective of this study was to assess the systemic toxic potential of Arabinofuranosidase, batch PPH40331 when administered orally by gavage to Han Wistar rats for 13 weeks.

The study was conducted according to GLP and in compliance with the OECD test guideline 408 (adopted in 1998): Repeated Dose 90-Day Oral Toxicity Study in Rodents.

Three groups, each comprising 10 males and 10 females, received doses of 10, 33 or 100% of Arabinofuranosidase, batch PPH40331 hereafter low, mid and high dose, respectively, at a dose volume of 10 mL/ kg body weight. A similarly constituted control group received the vehicle (reverse osmosis water) at the same volume dose.

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

General appearance and behaviour, sensory reactivity responses and motor activity were not affected by treatment and there were no deaths during the study. There was no effect of treatment on body weight gain or on food and water consumption. There were no treatment-related ophthalmic findings. Result of formaulations analysis demonstrate acceptable formulation.

The grip strength investigation in Week 12 did not identify any differences from controls that were attributable to treatment, however the hindlimb grip strength of females receiving mid or high dose were slightly higher than the controls, attaining statistical significance. The mean values for these groups were within the historical control range and the difference in females was attributed to a lower than expected group mean value in the control females which was just below the background range. In addition, the group mean forelimb grip strength values for males receiving low or mid dose were slightly lower than controls, attaining statistical significance, but the mean values were within the background range. There was no similar finding in the high dose males and the difference was attributed to higher than expected values for the control males that were above the background range. Consequently, these variations of grip strength in males and females were not attributable to treatment.

The haematology and blood chemistry investigations during Week 13 did not identify any toxicologically significant differences from controls. However, the haematological examination in Week 13 did show a small number differences from controls which attained statistical significance but these were minor, confined to one sex or lacked dose-relationship and were therefore attributed to normal biological variation. Such differences included a small reduction of reticulocyte count in high dose females but this did not associate with any other change in the erythrocyte indices (mean cell haemoglobin concentration was minimally high in these animals but this was also observed at the lower doses, with no dose-response). Differences also included decreases of lymphocyte count In high dose males and at all doses in females but there was no dose response in

females and in each case the difference from controls was minimal. The values for 9 out of 10 high dose males were within the background range, and the values for all females in all treatment groups were within the background range.
Variations of clotting times for both males and females in all groups, where prothrombin times tended to be increased and activated partial thromboplastin times tended to be decreased, were considered of no toxicological significance since the magnitude of the difference from controls was small (<2 seconds in the high dose animals) and there was, in most cases, no dose-response (the difference in PT for males did not show a dose-response while the decrease in APTT for males showed a dose-response, and the increase in PT for females did show a dose-response while the difference in APTT for females did not show a dose-response).

The biochemical analysis of blood plasma in Week 13 did not identify any toxicologically significant difference from controls. However, a number of inter-group differences from controls occurred, some of which attained statistical significance, but these were minor, confined to one sex or lacked dose-relationship and were therefore attributed to normal biological variation. Such differences included the statistically significant reduction of plasma alkaline phosphatase activities in high dose males but reductions of the plasma activities of this enzyme are of no toxicological importance.

Organ weights were considered unaffected by treatment. A number of inter-group differences from controls occurred, some of which attained statistical significance, but these were minor, confined to one sex or lacked dose-relationship and were therefore attributed to normal biological variation. Such differences included increased adjusted kidney and liver weights in high dose males where the increase were minimal, were not observed in females, and were therefore considered of no toxicological significance.

There were no treatment-related macroscopic or histopathological findings.

It is concluded that oral administration for 13 weeks with Arabinofuranosidase, batch PPH40331 to Han Wistar with was well-tolerated and did not cause any adverse change. In the absence of any adverse effect in this study, the no-observed adverse-effect level (NOAEL) was considered to be high dose (100% of the Arabinofuranosidase batch PPH40331, equivalent to 1116 mg TOS/kg/day or 3132 ARXU(M)DV/kg/day).

4. MUTAGENICITY

4.1 Bacterial Reverse Mutation assay (Ames test)

Arabinofuranosidase, PPH40331 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. Included were treatments at concentrations up to 5000 µg TOS/mL, (the maximum recommended concentration according to current regulatory guidelines). A modified 'treat and plate' methodology was used for all treatments in this study, as Arabinofuranosidase, PPH40331 was a high molecular weight protein which may provide significant levels of free histidine or tryptophan (which may cause artefacts through growth stimulation in a standard plate-incorporation methodology assay).

All Arabinofuranosidase, PPH40331 treatments in this study were performed using formulations prepared in water for irrigation (purified water). Calculations of all test article

concentrations stated in this report include a correction to account for Total Organic Solids (TOS) content of 10.7% w/w, using a correction factor of 9.35.

Experiment 1 treatments of all the tester strains were performed in the absence and in the presence of S-9, using final concentrations of Arabinofuranosidase, PPH40331 at 16, 50, 160, 500, 1600 and 5000 µg TOS/mL, plus vehicle and positive controls. Following these treatments, no evidence of toxicity was observed in any of the tester strains.

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 Arabinofuranosidase, PPH40331 approaching the maximum test concentration and considered therefore most likely to provide evidence of any mutagenic activity. Following these treatments there was again no clear evidence of toxicity 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 numbers of revertant colonies per plate all fell within acceptable ranges for vehicle control treatments, and were elevated by positive control treatments.

No clear and concentration-related increases in revertant numbers were observed, and no increases above ≥2-fold (in strains TA98, TA100 and WP2 *uvrA* pKM101) or ≥3-fold (in strains TA1535 and TA1537) were observed following treatement to Arabinofuranosidase, PPH40331 when compared to the concurrent vehicle control, for treatments of all the test strains, in the absence and presence of S-9.

It was concluded that Arabinofuranosidase, PPH40331 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.

4.2 *In vitro* Micronucleus Test In Cultured Human Lymphocytes

Arabinofuranosidase, batch PPH40331 was tested in an *in vitro* micronucleus assay using duplicate human lymphocyte cultures prepared from the pooled blood of two female 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 water for irrigation (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 48 hours following mitogen stimulation by phytohaemagglutinin (PHA). The test article concentrations for micronucleus analysis were selected by evaluating the effect of Arabinofuranosidase, batch PPH40331 on the replication index (RI).

Appropriate negative (vehicle) control cultures were included in the test system under each treatment condition. The proportion of micronucleated binucleate (MNBN) cells in the vehicle cultures fell within (or very close to) the 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.

The study was therefore accepted as valid.

Pulse (3+21 hour) treatment of cells with Arabinofuranosidase, batch PPH40331 in the absence and presence of a rat liver metabolic activation system (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 concentrations analysed. The single exception to this was observed at the lowest concentration scored post 3+21 hour +S-9 treatment (3000 µg TOS/mL) where one of the two replicate cultures exhibited a marginal increase above the normal range. However, this increase was small with the group mean MNBN cell frequency falling within normal and no such increases observed at higher Arabinofuranosidase, batch PPH40331 concentrations analysed (all replicate cultures). As such, this isolated statistical increase was not considered of biological importance.

Following extended (24+24 hour) treatment of cells in the absence if S-9, small but statistically significant increases in MNBN cell values were observed for all three concentrations analysed, though at all concentrations the MNBN cell values of both replicate cultures fell within normal ranges. In addition, it was noted that the statistical significance observed post 24+24 hour –S-9 treatment was set against a low concurrent vehicle control response (0.1% MNBN cells versus historical control range of 0.1 to 0.9% MNBN cells). For these reasons the weak statistical increases observed were not considered of biological importance.

It is concluded that Arabinofuranosidase, batch PPH40331 did not induce biologically relevant increases in micronuclei in cultured human peripheral blood lymphocytes following treatment in the absence and presence of a rat liver metabolic activation system (S-9). Concentrations were tested up to 5000 µg TOS/mL, a recommended regulatory maximum concentration for in vitro micronucleus assays.

5. REFERENCES

5.1 Study reports

Envigo: Study No.: XL41YM. Novozymes Reference No.: 20156085: Arabinofuranosidase, batch PPH40331: Toxicity Study by Oral Gavage Administration to Han wistar Rats for 13 Weeks. (July 2016). LUNA file: 2016-12177

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SUBMISSION END