ORIGINAL SUBMISSION

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE

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

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
000675	10/10/2016
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):* N/A

3b. For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd):* _____

PART II – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position
janet oesterling	regulatory affairs specialist III

Company *(if applicable)*: Novozymes

Mailing Address *(number and street)*: 77 Perry Chapel Church Rd

City	State or Province	Zip Code/Postal Code	Country
Franklinton	NC	27525	USA

Telephone Number	Fax Number	E-Mail Address
9194943187	N/A	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

Xylanase enzyme from Trichoderma reesei produced by Talaromyces leycettanus

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).*

The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following cGMP. This xylanase enzyme preparation is intended for use as a processing aid in the manufacture of food and food ingredients produced from cereal grains such as corn, wheat, barley, and oats. The enzyme can be used in various food industries including brewing, cereal beverage processing, potable alcohol processing, bakery applications and grain processing (including corn wet milling). The maximum recommended use level is 650 FXU(S) per kg of dry matter. This equates to 0.715 mg TOS per kg of body weight per day. No special population is targeted.

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

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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	Xylanase	EC	3.2.1.8		
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.

Classification: Xylanase
IUBMB nomenclature: endo-1,4-β-xylanase
EC No.: 3.2.1.8
CAS No.: 9025-57-4
Specificity: Hydrolyze internal glucosidic β-1,4 bonds in xylan
Xylanases catalyse the endo-hydrolysis of 1,4-β-D-xylosidic linkages in xylan including arabinoxylan (also called pentosans). The enzymatic hydrolysis of arabinoxylans results in the generation of 1,4-beta-D-arabinoxylan oligosaccharides of variable lengths.

3. Synonyms

Provide as available or relevant:

1	endo-1,4-β-xylanase
2	
3	

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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 janet oesteling
(name of notifier)

has concluded that the intended use*(s)* of Xylanase enzyme from Trichoderma reesei produced by Talaromyces leycettanus
(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. ☒ janet oesteling *(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.

janet oesteling *(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.

(address of notifier or other location)

janet oesteling *(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: 2016.10.10 11:13:26 -04'00'	Janet Oesterling, Regulatory Specialist III	10/10/2016

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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)
	Form3667_xylanase from t. leycettanus by t. reesei_2016-09-30.pdf	Incoming Correspondence/Submission Form
	Claimletter_XylanasefromT.reesei_2016-10-10.pdf	Administrative
	DecisionTree_Xylanase from T. reesei_2016-10-10 .pdf	Administrative
	GRASNotification_Xylanasefrom T. reesei_2016-10-10.pdf	Submission
	Safetyof MicrobialEnzymePreps_ParizaandJohnson_April2001.pdf	Administrative
	SummaryofToxicityData_Xylanase_2016-09-30.pdf	Administrative

novozymes®
Rethink Tomorrow

October 10, 2016

RE: GRAS Notification - Exemption Claim

Dear Sir or Madam:

Pursuant to the proposed 21C.F.R.§ 170.36 (c)(1) Novozymes North America Inc. hereby claims that xylanase preparations produced by submerged fermentation of a genetically modified *Trichoderma reesei* are Generally Recognized as Safe; therefore, they are exempt from statutory premarket approval requirements.

The following information is provided in accordance with the proposed regulation:

Proposed § 170.36 (c)(1)(i) *The name and address of the notifier.*

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

Proposed § 170.36 (c)(1)(ii) *The common or usual name of notified substance.*

Xylanase Enzyme from *Talaromyces leycettanus* Produced by *Trichoderma reesei.*

Proposed § 170.36 (c)(1)(iii) *Applicable conditions of use*.

This xylanase enzyme preparation is intended for use as a processing aid in the manufacture of food and food ingredients produced from cereal grains such as corn, wheat, barley, and oats. The enzyme can be used in various food industries including brewing, cereal beverage processing, potable alcohol processing, bakery applications and grain processing (including corn wet milling) 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.

Proposed §170.36 (c)(1)(iv) *Basis for GRAS determination*.

This GRAS determination is based on scientific procedures.

Proposed § 170.36 (c)(1)(v) *Availability of information*.

A notification package providing a summary of the information which supports this GRAS determination is enclosed with this letter. The package includes a safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure. Complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying at reasonable times at Novozymes North America, Inc. or will be sent to FDA upon request.

(b) (6)

_____ 10-10-16_
Janet Oesterling Date
Regulatory Affairs Specialist III

Xylanase from *Talaromyces leycettanus* produced by *Trichoderma reesei*

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

October 2016

1. GENERAL INTRODUCTION

The subject of this notification is a xylanase enzyme preparation produced by submerged fermentation of a genetically modified *Trichoderma reesei* microorganism carrying the gene coding for xylanase from *Talaromyces leycettanus.*

This xylanase enzyme preparation is intended for use as a processing aid in the manufacture of food and food ingredients produced from cereal grains such as corn, wheat, barley, and oats. The enzyme can be used in various food industries including brewing, cereal beverage processing, potable alcohol processing, bakery applications and grain processing (including corn wet milling) by contributing to the degradation of the plant's cell wall matrix.

The active enzyme is xylanase (EC 3.2.1.8, CAS 9025-57-4).

The information provided in the following sections is the basis for our determination of general recognition of safety of the xylanase enzyme preparation. Our safety evaluation in Section 7 includes an evaluation of the production strain, the donor strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure to the preparation.

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 (1) (2) (Appendix 1). The production organism for the xylanase, *Trichoderma reesei,* is discussed in Sections 2 and 7. 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 (3) (4). 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 (3) (4).

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 (5) (6) (7) (8) (9) (10). The methods used to develop the genetically modified production organism and the specific genetic modifications introduced into the production organism are described in Section 2.

This notification includes information that addresses the safety of the enzyme source, the enzyme component, the manufacturing process and a consideration of dietary exposure which covers all the issues relevant to a safety evaluation of an enzyme preparation. Based on critical review and evaluation of its published and unpublished

information, Novozymes concludes through scientific procedures that the subject of this notification, xylanase 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.

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The *Trichoderma reesei (T. reesei)* production strain, designated TrGMEr83-53a1, was derived from recipient strain BTR213, a natural isolate of *T. reesei* strain RUTC30 (ATCC 56765). RUTC30 is derived from the well-known wild type strain QM6a. QM6a is the parent of practically all *T. reesei* industrial production strains (3). *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 (11).

T. reesei complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms (7). It also meets the criteria for a safe production microorganism as described by Pariza and Foster (2) and later Pariza and Johnson (1) and several expert groups (12) (3) (4) (13).

The expression plasmid, used in the strain construction, pAmFs250, contains strictly defined chromosomal DNA fragments and synthetic DNA linker sequences. The DNA sequence for the introduced gene is based on the *xynTL* sequence encoding a xylanase from *Talaromyces leycettanus*.

2.2 Recipient Strain

The recipient strain BTR213 used in the construction of the xylanase production strain was modified by several rounds of classical mutagenesis of RUTC30, screening for increased level of enzyme production.

2.3 Xylanase Expression Plasmid

The expression plasmid, pAmFs250, used to introduce the *xynTL* 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 *xynTL* gene encoding the xylanase, 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.

2.4 Construction of the Recombinant Microorganism

The production strain, *Trichoderma reesei* TrGMEr83-53a1, was constructed from the recipient strain BTR213 through the following steps:

1. The expression cassette from plasmid pAmFs250 was integrated into one specific locus in strain BTR213 by targeted homologous recombination to this locus. Targeted integration allows the expression of the *xynTL* gene from the promoter.

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

The resulting xylanase production strain containing one copy of the *xynTL* gene at the target locus was named TrGMEr83-53a1.

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

2.5 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 a xylanase gene specific probe showed an identical band pattern compared to the reference production strain (TrGMEr83-53a1), 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.

2.6 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.

2.7 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 (5) is satisfactorily addressed.

3. MANUFACTURING PROCESS

This section describes the manufacturing process for the xylanase which follows standard industry practices (14) (15) (16). The quality management system used in the manufacturing process for the xylanase complies with the requirements of ISO 9001. It is produced under a standard manufacturing process as outlined by Aunstrup (15) 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. The enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in the Food Chemicals Codex (17). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (18).

3.1 Raw Materials

The raw materials used in the fermentation and recovery process for the xylanase enzyme concentrate are standard ingredients used in the enzyme industry (14) (15) (16). 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 if used in the product is less than 1%.

3.2 Fermentation Process

The xylanase enzyme preparation is produced by pure culture submerged fed-batch fermentation of a genetically modified strain of *T. reesei* as described in Section 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.

3.2.1 Production Organism

Each batch of the fermentation process is initiated with a stock culture of the production organism, *T. reesei,* described in Section 2. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

3.2.2 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.

3.3 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.

3.3.1 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

3.3.2 Formulation and Standardization Processes

The liquid enzyme preparation is standardized with sucrose and preserved with potassium sorbate and sodium benzoate. See Table 1 below.

3.4 Quality Control of Finished Product

The final products are analyzed according to the specifications given in Section 5.

4. ENZYME IDENTITY

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

Classification	Xylanase
IUBMB nomenclature:	endo-1,4-β-xylanase
EC No.:	3.2.1.8
CAS No.:	9025-57-4
Specificity:	Hydrolyze internal glucosidic β-1,4 bonds in xylan
Amino acid sequence:	the total nucleotide and amino acid sequences have been determined

5. COMPOSITION AND SPECIFICATIONS

5.1 Quantitative Composition

The xylanase 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.

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%
Water	45 - 65%

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

5.2 Specifications

The xylanase enzyme preparation complies with the recommended purity criteria for enzyme preparations as described in *Food Chemicals Codex* (17). 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 (18).

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	PPQ40405	PPQ40242	PPQ40356
Activity unit	FSU(S)/g	1510	1540	1600
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

6. APPLICATION

6.1 Mode of Action

The active enzyme is a xylanase (EC 3.2.1.8). Xylanases catalyse the endo-hydrolysis of 1,4-β-D-xylosidic linkages in xylan including arabinoxylan (also called pentosans). 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 xylanase preparation is used during food processing to aid in the separation of grains into the germ, starch, gluten and fibers.

6.2 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.

Brewing and Cereal Beverage Processes: Up to 500 FXU(S) per kg starch dry matter.

Baking and other Cereal Food Processes: Up to 650 FXU(S) per kg of flour

Grain processing: Up to 477 FXU(S) per kilo of corn dry matter.

6.3 Enzyme Residues in the Final Food

Xylanases catalyse the endo-hydrolysis of 1,4-β-D-xylosidic linkages in xylan including arabinoxylan (also called pentosans). The enzymatic hydrolysis of arabinoxylans results in the generation of 1,4-beta-D-arabinoxylan oligosaccharides of variable lengths. Like the substrate, these oligosaccharides are natural constituents of cereal-containing foodstuffs.

The enzyme used in the foodstuffs is largely heat inactivated during processing. The reasons why enzymes do not exert any (unintentional) enzymatic activity in the final

food are a combination of various factors, depending on the application and the process conditions used by the individual food producer. These factors include denaturation of the enzymes during processing, depletion of the substrate, lack of water activity, wrong pH, etc. In some cases the enzymes may no longer be present in the final food, due to the removal during processing. Therefore, the enzyme does not exert a function in the final food/beverage.

7. SAFETY EVALUATION

7.1 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 (2) (1). 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 (19). Pariza and Foster (2) 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 30 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 (20). 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 (3) (4). *Trichoderma reesei* strains are non-pathogenic for healthy humans and animals (3). The safety of *T. reesei* has been discussed in several review papers (3) (4) (21) (22). *Trichoderma 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) (21). However, the bulk of the literature investigating the capability of *T. reesei* to produce peptaibols 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 (23) (24) (21).

In 2012, the US EPA published a risk assessment (25) 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 this production strain (TrGMEr83-53a1).

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 (26). 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 (2) and further developed by IFBC in 1990 (19), the EU SCF in 1991 (6), the OECD in 1992 (7), ILSI Europe Novel Food Task Force in 1996 (10), FAO/WHO in 1996 (9), JECFA in 1998 (18) and Pariza and Johnson in 2001 (1), 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 Section 2 and 3.

Novozymes' used the decision tree (Appendix 2) in Pariza and Johnson 2001 (1) as a basis for our safety assessment. The production strain is genetically modified as discussed in Section 2. The expressed enzyme product is xylanase. 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.

7.2 Safety of the Donor Organism

The donor organism of the xylanase is *Talaromyces leycettanus*. As indicated in Section 2 the introduced DNA is well defined and characterized. Only well characterized DNA fragments, limited solely to the xylanase 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.

7.3 Safety of the Xylanase Enzyme

As indicated in section 4, the subject of this GRAS notification is a xylanase, EC 3.2.1.8. Enzymes including xylanase have a long history of use in food (1) (2) and animal feed (27). Xylanase has been used extensively for more than 25 years in various industrial food applications such as starch processing, manufacturing of alcohol, brewing and baking products (28).

Enzyme proteins do not generally raise safety concerns (1) (2) . Pariza and Foster (2) note that very few toxic agents have enzymatic properties. The safety of the xylanase was assessed using the Pariza and Johnson, (2001) decision tree (Appendix 2).

Based on the information above, it is concluded that xylanase enzymes have a history of safe use in food and do not have toxic properties.

7.3.1 Allergenic Potential of the Xylanase 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).

In order to further evaluate the possibility that the xylanase 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 (30) and modified by Codex Alimentarius

Commission, 2009 (31) the xylanase 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**).

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 xylanase 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 xylanase 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.

On the basis of the available evidence it is concluded that oral intake of xylanase produced by *T. reesei* is not anticipated to pose any food allergenic concern.

7.4 Safety of the Manufacturing Process

The xylanase 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 (14) (15) (16).

7.5 Safety studies

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

7.5.1 Description of Test Material

The following studies were performed on test batch PPQ40100 with favourable results:

- Reverse Mutation Assay (Ames test)
- In vitro Cytotoxicity Test: Neutral Red Uptake
- In vitro Cytogenecity Test: Micronucleus Assay
- 13 week sub-chronic oral toxicity study

These tests are described in Appendix 3. Based on the present toxicity data and the history of safe use for the strain, it can be concluded that xylanase batch PPQ40100 shows no treatment-related toxicological effects in vivo and does not exhibit genotoxicological potential in the guideline directed genotoxicological in vitro assays.

7.6 Estimates of Human Consumption and Safety Margin

The xylanase enzyme is to be used in a variety of applications. And, as mentioned in Section 6.3, when using the food enzyme in food and brewing processes the enzyme will not be functional in the final product due to the application process conditions used by the individual food producer.

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 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 which represents a "maximum worst case" situation of human consumption. Overall, the human exposure to the xylanase 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.

The xylanase has an average activity of 1550 FXU(S)/g and approximately 11% TOS (Total Organic Solids) content.

This corresponds to an activity/TOS ratio of 14.09 FXU(S)/mg TOS.

Assumptions in the Budget Method

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

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

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

It is further assumed that all processed beverages contain 10% hydrolysed starch dry matter = 2.5 g hydrolysed starch derived dry matter per kg body weight per day.

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

Intake associated with grain processing, baking and other cereal based food processes:

TMDI: Grain Processing

The maximum recommended dosage in grain processing is: 477 FXU(S)/kg corn dry 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:

477 FXU(S)/kg corn dry matter ÷ 0.70 kg starch in corn dry matter = 681 FXU(S)/kg starch dry matter.

This corresponds to 48.32 mg TOS

The theoretical maximum daily intake (TMDI) of consumers of the food enzyme based on the starch contribution is therefore:

48.33 mg TOS/kg ÷ 1000 g/kg x (3.12 + 2.50 = 5.62) g = 0.272 mg TOS/kg body weight/day.

TMDI: Baking and other Cereal Based Food Processes

The maximum recommended dosage in bakery and other cereal based food processing is: 650 FXU(S)/kg of starch dry matter.

This corresponds to 46.10 mg TOS

The theoretical maximum daily intake (TMDI) of consumers of the food enzyme based on the starch contribution is therefore:

46.10 mg TOS ÷ 1000 g/kg x (3.12 + 2.50 = 5.62) g = 0.260 mg TOS/kg/kg body weight/day

Intake associated with beer and other cereal based beverage processes:

In order to demonstrate a worst case calculation, an exaggerated human intake for beer and beer-like beverages was used. This intake calculation is based on a mean and the 90th percentile consumption of alcoholic beverages in the United States using NHANES Survey 2003-2012 combined 2-day consumption data (32), for a 60 kg person. Thus, using the highest mean intake of beer and the lowest weight average represents the "worst case" scenario.

Based on this, 15.8 g of beer and beer-like beverage is consumed kg of body weight per day.

Typical values for the starch content of malt and barley is 65% (33). As a rule of thumb 1 kg of grits will be used for the production of 6 kg of beer. Therefore, an intake per kg bw per day of 15.8 g "Beer and beer-like beverage" corresponds to:

15.8 g beer/kg bw/day ÷ 6 g beer/g grits 2.63 g grits/kg bw/day x 0.65 g starch/per g grits = 1.71g starch/kg bw/day.

TMDI: Brewing and Cereal Based Beverages

The maximum recommended dosage for brewing processes and other cereal based beverage processes is 500 FXU(S)/kg starch.

This corresponds to 35.49 mg TOS

The theoretical maximum daily intake (TMDI) of consumers of the food enzyme based on the starch contribution is therefore:

35.49mg TOS/kg starch x 1.71g starch/kg bw/day ÷ 1000 = 0.183mg TOS/kg bw/day

TMDI: Grain, Brewing and Baking Processes:

The theoretical maximum daily intake (TMDI) of consumers of the food enzyme based on both starch and brewing processing is:

(0.272 + 0.183 + 0.260) mg TOS/kg bw/day = 0.715 mg TOS/kg bw/day

The TMDI contribution from beverage alcohol is assumed to be zero due to the distilling process.

7.6.2 Safety margin

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 xylanase, PPQ40100 was the highest dosage possible, 1051 mg TOS/kg bw/day. See Appendix 3.

See Table 3 below.

Table 3. NOAEL Calculation

NOAEL (mg TOS/kg bw/day)	1051
*TMDI (mg TOS/kg bw/day)	0.715
Safety margin	1469

*based on the worst case scenario

8. Results and Conclusions

On the basis of the evaluation contained in Section 7, a review of the published literature, the history of safe use of *T. reesei* and the limited and well defined nature of the genetic modifications, the xylanase enzyme preparation is safe for its intended use.

9. List of 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

3. Summary of Toxicity Data. August 2016, LUNA No. 2016-12578-01.

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Appendix 2- Pariza & Johnson Decision Tree analysis of a Xylanase from *Talaromyces leycettanus* produced by *Trichoderma reesei*

This Xylanase from *Talaromyces leycettanus* 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
   The
   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.

Toxicology & Product Safety

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SUMMARY OF TOXICITY DATA

Xylanase, batch PPQ40100 from *Trichoderma reesei*

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1. ABSTRACT

The below series of toxicological studies were undertaken to evaluate the safety of Xylanase, batch PPQ40100.

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 August 2016.

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

- Xylanase, batch PPQ40100 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 1051 mg TOS/kg/day and 14470 FXU(S)/kg/day.

- It was concluded that Xylanase, batch PPQ40100 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 presence of a rat liver metabolic system (S-9), and using a modified 'Treat and Plate' methodology.

- Xylanase, batch PPQ40100 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). 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 Xylanase, represented by batch PPQ40100, exhibits no significant toxicological effects under the experimental conditions described.

2. TEST SUBSTANCE

Xylanase (IUBMB/E.C. 3.2.1.8) catalyzes the hydrolysis of linear polysaccharide to xylose.The enzyme is used in the corn industry to increase starch and gluten yields during wet milling.

2.1 Characterization

The batch Xylanase, batch PPQ40100 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 Xylanase, batch PPQ40100

Batch number	PPQ40100
Activity (FXU(S)/g)	1390
BCA_Total mg/g	107.7
BCA(B) mg/g	96.2
N-Total (% w/w)	1.23
Water (KF) (% w/w)	89.4
Dry matter (% w/w)	10.6
Ash (% w/w)	0.5
Total Organic Solids (TOS[1]) (% w/w)	10.1
Specific gravity (g/mL)	1.041

[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 Xylanase, batch PPQ40100 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 Xylanase, batch PPQ40100 hereafter named 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 daily visual observation), ophthalmic examination, hematology (peripheral blood), blood chemistry, organ weight, macropathology and histopathology investigations were undertaken.

General appearance and behaviour, sensory reactivity responses, grip strength and motor activity were not affected by treatment. There was one premature death during the study, but this was attributed to a traumatic injury and was not associated with treatment. There was no effect of treatment on body weight gain or on food and water consumption. There were no treatment-related ophthalmic findings. The hematology and blood chemistry investigations during Week 13 did not identify any toxicologically significant differences from controls. Organ weights were unaffected by treatment and there were no treatment-related macroscopic or histopathological findings.

It is concluded that oral administration of Xylanase, batch PPQ40100 to Han Wistar rats at doses up to 100% of the Xylanase, batch PPQ40100 for 13 weeks was well-tolerated and did not cause any adverse change. The no-observed adverse-effect level (NOAEL) was considered to be 100% of the Xylanase, Batch PPQ40100 (equivalent to 1051 mg TOS/kg/day or 14470 FXU(S)/kg/day).

4. MUTAGENICITY

4.1 Bacterial Reverse Mutation assay (Ames test)

Xylanase, batch PPQ40100 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 Xylanase, batch PPQ40100 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 Xylanase, batch PPQ40100 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 10.1% w/w, using a correction factor of 9.9.

Experiment 1 treatments of all the tester strains were performed in the absence and presence of S-9 using final (nominal) concentrations of Xylanase, batch PPQ40100 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, as would normally be manifest as a thinning of the background bacterial lawn and/or a marked reduction in revertant numbers.

Experiment 2 treatments of all the tester strains were performed in the absence and 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 Xylanase, batch PPQ40100 approaching the maximum test concentration and therefore considered 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 indication of any test article related amino acid feeding effects were observed on any of the test plates, as would be manifest by a thickening of the background bacterial lawn.

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 Xylanase, batch PPQ40100 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 Xylanase, batch PPQ40100 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

Xylanase, batch PPQ40100 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 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 Xylanase, batch PPQ40100 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 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 Xylanase, batch PPQ40100 in the absence and presence of S-9 resulted in frequencies of micronucleated binucleate (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 a single replicate culture at the lowest and intermediate concentrations analysed post 3+21 hour +S-9 treatment, the MNBN cell frequency of all treated cultures (all concentrations) fell within the normal range. There were no indications of any test article concentration related effect on MNBN cell frequency (negative Cochran-Armitage trend).

It is concluded that Xylanase, batch PPQ40100 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). Concentrations were tested up to 5000 µg TOS/mL, a recommended regulatory maximum concentration for *in vitro* micronucleus assays.

5. STUDY REPORT

Envigo: Study No.: DK60TW. Novozymes Reference No.: 20156084 Xylanase, batch PPQ40100: Toxicity Study by Oral Gavage Administration to Han wistar Rats for 13 Weeks. (August 2016). LUNA file: 2016-13068

Covance: Study No.: 8336499. Novozymes Reference No.: 20166003. Xylanase, batch PPQ40100: Bacterial Reverse Mutation Assay using Treat and Plate Modification. (May 2016). LUNA file: 2016-08692.

Covance: Study No.: 8336501. Novozymes Reference No.: 20166004: Xylanase, batch PPQ40100: *In Vitro* Human Lymphocyte Micronucleus Assay. (April 2016). LUNA file: 2016-06922

SUBMISSION END