Xylanase Enzyme Produced by *Bacillus licheniformis*

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

January 2022

PART 1: SIGNED STATEMENT AND CERTIFICATION

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

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

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

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

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

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

The appropriately descriptive term for this notified substance is xylanase enzyme preparation produced by *Bacillus licheniformis*

§170.225(b) – Trade secret or confidential:

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

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

The xylanase enzyme preparation is used as a processing aid during food manufacturing to aid in the separation of grains into the germ, starch, gluten and fiber. The enzyme can be used in any food application where the starch that is present can be modified by the xylanase. Some examples of these applications include starch processing, brewing and other cereal based beverage processes. 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.

01/13/2022

Janet Oesterling — Date
Sr. Regulatory Affairs Specialist

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

2.1 IDENTITY OF THE NOTIFIED SUBSTANCE

The subject of this notification is a xylanase enzyme produced by submerged fermentation of a genetically modified *Bacillus licheniformis* microorganism carrying the gene coding for xylanase from *Chryseobacterium sp-10696.*

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

Classification:	xylanase
IUBMB nomenclature:	endo-1,4-beta-xylanase
EC No.:	3.2.1.8
CAS No.:	9025-57-4
Specificity:	hydrolyzes internal glucosidic β-1,4 bonds in arabinoxylan
kDA:	60.8
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 *Bacillus licheniformis* production strain, designated SJ14481, was constructed via the intermediate strain MDT223. MDT223 is derived from a natural isolate of *Bacillus licheniformis* strain DSM 9552. *B. licheniformis* is generally regarded as non-pathogenic and is widely distributed in nature. It is classified as a Class 1 organism according to the NIH guidelines (1). Risk Group 1 organisms are those not associated with disease in healthy adult humans.

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

The expression plasmid used in the strain construction, contains strictly defined chromosomal DNA fragments and synthetic DNA linker sequences. The donor for the modified xylanase is *Chryseobacterium sp-10696*.

2.2(b) Recipient Strain

The recipient strain used in the construction of the xylanase production strain was modified at several chromosomal loci during strain development to inactivate genes encoding a number of proteases. Also, deletion of a gene essential for sporulation was performed, eliminating the ability to sporulate, together with the deletion of additional genes encoding unwanted proteins that can be present in the culture

supernatant. The lack of these represents improvements in the product purity and stability.

2.2(c) Xylanase Expression Plasmid

The expression plasmid used to transform the Bacillus licheniformis recipient strain is based on the well-known *Bacillus* vectors pE194 (8) and pUB110 (9) from Staphylococcus aureus. No elements of these vectors are left in the production strain. The plasmid contains the expression cassette consisting of a *B. licheniformis* promotor, the *xylCB-0087* sequence encoding the modified xylanase and a transcriptional terminator.

Only the expression cassette with elements between the promoter fragment and the terminator are present in the final production strain. This has been confirmed by whole genome sequencing.

2.2(d) Construction of the Recombinant Microorganism

In the construction of the production strain, *Bacillus licheniformis* SJ14481 the expression cassette was integrated into two loci by targeted homologous recombination. Targeted integration of the expression cassette at these loci allows the expression of the xylanase gene *xylCb-0087* from the promoter.

The resulting xylanase strain containing two copies of the *xylCb-0087* gene was named SJ14481.

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

2.1(e) Stability of the Introduced Genetic Sequences

The xylanase gene *xylCb-0087* is integrated into the *Bacillus licheniformis* chromosome. Thus, it is as such poorly mobilized for genetic transfer to other organisms and is considered mitotically stable. The phenotypic and genetic stability of the *Bacillus licheniformis* is proven by its capacity to produce a constant level of the xylanase enzyme. This was assessed by measuring the enzyme activity in three independent batches of the food enzyme, as outlined in Table 2 below.

Furthermore, the protein spectrum for 3 batches showed identical expression profiles. Thus, stable enzyme production of the desired enzyme combined with the identical protein expression profile in 3 batches confirm the stability of the *Bacillus licheniformis* production strain

2.2(f) Antibiotic Resistance Gene

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

2.2(g) Absence of Production Organism in Product

The absence of the production organism is an established specification for the commercial product. The production organism does not end up in food and therefore the first step in the safety assessment as described by IFBC (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 xylanase enzyme 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 xylanase enzyme preparation is produced by pure culture submerged fed-batch fermentation of a genetically modified strain of *Bacillus licheniformis* 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, *Bacillus licheniformis,* described in Part 2. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

2.3(d) Criteria for the Rejection of Fermentation Batches

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

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

1) Contamination is observed in 2 or more samples by microscopy
2) Contamination is observed in two successive agar plates at a minimum interval of 6 hours

Any contaminated fermentation is rejected.

2.3(e) Recovery Process

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

2.3(f) Purification Process

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

1) Pretreatment - pH adjustment and flocculation (if required)
2) Primary Separation – vacuum drum filtration or centrifugation
3) Concentration - ultrafiltration and/or evaporation
4) Pre- and Germ Filtration - for removal of residual production strain organisms and as a general precaution against microbial degradation
5) Final concentration – evaporation and/or ultrafiltration.
6) Preservation and Stabilization of the liquid enzyme concentrate

The enzyme concentrate is stabilized with sorbitol. The liquid product is further 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 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. 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
Water	45 - 65%
Sorbitol	45 - 50%
Enzyme Solids (TOS*)	7.4%
Sodium Benzoate	<0.5%
Potassium Sorbate	<0.5%

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

2.4(b) Specifications

The xylanase 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

Xylanase activity unit	FXU(TB)/g	472	541	383
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 endo-1, 4-beta-xylanase (EC 3.2.1.8), which hydrolyses xylosidic linkages in the arabinoxylan backbone resulting in a depolymerization of the arabinoxylans into smaller oligosaccharides. Arabinoxylans are highly branched xylans

that are characteristic for the outer cell walls and endosperm of cereals such as 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 fiber.

The enzyme is primarily used in industries processing plant cell walls, where it contributes to degradation of the plant cell wall matrix.

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.

Grain processing: Up to 200 FXU(TB) per kilo of corn dry matter.

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

2.5(c) Enzymes Residues in the Final Food

The xylanase enzyme that is 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.

Estimates of the potential enzyme exposure are set forth in the following sections.

PART 3 - DIETARY EXPOSURE

The xylanase will be used in a variety of applications. As mentioned previously, when using the enzyme in food applications the enzyme will not be functional in the final food 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 general population is the target population for consumption. There is no specific subpopulation.

3(a) Estimates of Human Consumption and Safety Margin

The assumptions are highly exaggerated since the enzyme protein and the other compositional substances are diluted or removed during certain processing steps. Furthermore, all processed foods and beverages produced with the enzyme are not always produced with the maximum recommended dosage. Overall, the human exposure to the xylanase enzyme will be negligible. 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 exaggerated.

3(b) Food Consumption Data

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 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.

Assumption in the Budget Method

The xylanase concentrate has an average activity of 465 FXU(TB)/g and approximately 6.4% TOS (Total Organic Solids) content.

This corresponds to an activity/TOS ratio of 7.3 FXU(TB)/mg TOS.

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 day. Assuming that 25% of the non-milk beverages are processed, the daily consumption will be 25 ml processed beverages per kg body weight.

It is further assumed that all processed beverages contain 10% starch (or starch-derived) dry matter = 2.5 g starch derived dry matter per kg bw per day.

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

TMDI: Grain Processing

The maximum recommended dosage in grain processing is: 200 FXU(TB)/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:

200 FXU(S)/kg corn dry matter ÷ 0.70 kg starch in corn dry matter = 286 FXU(TB)/kg starch dry matter.

This corresponds to 39.2 mg TOS/kg

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

39.2 mg TOS/kg ÷ 1000 g/kg x (3.12 + 2.50 = 5.62) g = 0.22 mg TOS/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 (17), 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% (18). 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 = 1.71 g 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(TB)/kg starch.

This corresponds to 80 mg TOS

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

68.5 mg TOS/kg starch x 1.71 g starch/kg bw/day ÷ 1000 = 0.12 mg TOS/kg bw/day

Total TMDI from applications above: 0.34 mg TOS/kg bw/day

Theoretical Maximum Daily Intake (TMDI)

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

The NOAEL dose level in the 13-week oral toxicity study in rats conducted on amylase tox batch PPQ69045 was the highest dosage possible, 962 mg TOS/kg bw/day.

The safety margin is calculated as dose level with no adverse effect (NOAEL) divided by the estimated human consumption. See the Summary of Toxicity Data attached as an appendix and Table 3 below.

Table 3. NOAEL Calculation

NOAEL (mg TOS/kg bw/day)	962
*TMDI (mg TOS/kg bw/day)	0.34
Safety margin	2829

*based on the worst-case scenario

PART 4 - SELF-LIMITING LEVELS OF USE

This part does not apply

PART 5 - COMMON USE IN FOOD BEFORE 1958

This part does not apply

PART 6 - NARRATIVE ON THE CONCLUSION OF GRAS STATUS

The information provided in the following sections is the basis for our conclusion of the general recognition of safety for the xylanase enzyme preparation. The evaluation follows the generally recognized methodology and the decision tree by Pariza and Johnson 2001 (19). Our safety evaluation in Part 6 follows the approach described in the Enzyme Technical Association publication (Sewalt et al 2016) (20) which includes an evaluation of the production organism, the donor strain, the introduced DNA, the enzyme and the manufacturing process. Data and information cited in this notification is generally available and Part 6 does not contain any data or information that is exempt from disclosure under the FOIA.

6(a) Decision Tree

This xylanase enzyme preparation produced by Bacillus licheniformis was evaluated according to the decision tree published in Pariza and Johnson, 2001 (4). The result of the evaluation is presented below in the Decision Tree.

1. Is the production strain genetically modified?
   YES
   If yes, go to 2.

2. Is the production strain modified using rDNA techniques?
   YES
   If yes, go to 3**.**

3. Issues relating to the introduced DNA are addressed in 3a-3e.

   3a. Does the expressed enzyme product which is encoded by the introduced DNA have a history of safe use in food?
   YES
   If yes, go to 3c.

   3c. Is the test article free of transferable antibiotic resistance gene DNA?
   YES
   If 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
   If yes, go to 4.

4. Is the introduced DNA randomly integrated into the chromosome?
   NO
   If no, go to 6.

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeatedassessment via this evaluation procedure?
YES

Test article is accepted

6(b) Safety of the Manufacturing Process

This Part describes the manufacturing process for the xylanase, which follows standard industry practices (13) (11) (12).

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

The enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in the Food Chemicals Codex (21). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (15).

6(c) Safety of the Production Organism

The safety of the *Bacillus lichiformis* production organism must be the prime consideration in assessing the degree of safety of an enzyme preparation intended for use in food (22) (4).

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 (23). Pariza and Foster (3) 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”.

Bacillus licheniformis is generally regarded as non-pathogenic and is widely distributed in nature. It is a Class 1 organism according to the NIH guidelines: *Guidelines for Research Involving Recombinant DNA Molecules, Federal Register, Dec.19, 2001 (66 FR 57970)*. Risk Group 1 organisms are those not associated with disease in healthy adult humans.

The *Bacillus licheniformis* production strain is genetically modified by rDNA techniques. The methods used to develop the genetically modified production organism and the specific genetic modifications introduced into the production organism are described in Part 2.

The enzyme preparation is free of DNA encoding transferable antibiotic resistance gene DNA. The introduced DNA is well characterized and safe for the construction of microorganisms to be used in the production of food grade products. The DNA is stably integrated into the chromosome at specific sites in the chromosome and the

incorporated DNA is known not to encode or express any harmful or toxic substances.

Bacillus licheniformis has a long history of safe industrial use in the production of enzymes used in human food. It is widely recognized as a harmless contaminant found in many foods (23). *Bacillus licheniformis* is not a human pathogen and it is not toxigenic (24).

Bacillus licheniformis is been used in the fermentation industry for the production of enzymes, antibiotics, and other specialty chemicals. Various enzymes are produced by *Bacillus licheniformis* and are considered GRAS substances (GRNs 265, 277, 472, 572, 587, 645) (25).

In addition, it has also been granted a Qualified Presumption of Safety status by the European Food Safety Authority (26).

6(d) Safe Strain Lineage

The safety of this *Bacillus licheniformis* production strain was established following published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients (19) (23). The *Bacillus licheniformis* production strain is derived from a safe strain lineage that is comprised of production strains for enzyme preparations which have full toxicological safety studies (i.e. 13-week oral toxicity study in rats, Ames test and chromosomal aberration test or micronucleus assay).

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

Table 4: Safe Strain Lineage

Enzyme	EC No.	Predecessor strain[1]	Donor strain	Safety studies[2]
Alpha-amylase (GRASP 0G0363)	3.2.1.1	*Bacillus licheniformis* Si3	*Bacillus stearothermophilus*	Yes
Alpha-amylase (GRN 22)	3.2.1.1	*Bacillus licheniformis* SJ1707	*Bacillus licheniformis*	Yes
Cyclodextrin glucanotransferase	2.4.1.19	*Bacillus licheniformis* SJ1707	*Thermoanaerobacter sp.*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* SJ1707	*Bacillus licheniformis*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* SJ1904	*Bacillus licheniformis*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* MDT223	*Bacillus stearothermophilus*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* MDT223	*Bacillus amyloliquefaciens*	Yes
Serine protease (GRN 564)	3.4.21.1	*Bacillus licheniformis* MDT223	*Nocardiopsis prasina*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* MDT223	*Bacillus licheniformis*	Yes
Xylanase (GRN 472)	3.2.1.8	*Bacillus licheniformis* MDT223	*Bacillus licheniformis*	Yes
L-Glutaminase (GRN774)	3.5.1.2	*Bacillus licheniformis MDT223*	*Bacillus licheniformis*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis PP3579*	*Bacillus licheniformis*	Yes

Beta-amylase	3.2.1.1	*Bacillus licheniformis* PP3579	*Bacillus flexus*	Yes
Beta-galactosidase (GRN 572)	3.2.1.23	*Bacillus licheniformis* AEB1763	*Bifidobacterium bifidum*	Yes
Acetolactate decarboxylase (GRN 587)	4.1.1.5	*Bacillus licheniformis* AEB1763	*Bacillus brevis*	Yes
Pullulanase (GRN 645)	3.2.1.41	*Bacillus licheniformis* AEB1763	*Bacillus deramificans*	Yes
PI-phospholipase C (GRN 728)	3.1.4.11	*Bacillus licheniformis* AEB1763	*Pseudomonas sp-62186*	Yes
Phospholipase-C (GRN 689)	3.1.4.3	*Bacillus licheniformis AEB1763*	*Bacillus thuringiensis*	Yes

Table 4. Novozymes products derived from *B. licheniformis* strains. 1The predecessor strains show common strains in the GM construction pathway. 2At least the following: in vitro test for gene mutations in bacteria (Ames); in vitro test for chromosomal aberration or in vitro micronucleus assay; 13-week sub chronic oral toxicity study in rats. The conclusions of these studies were in all cases favorable.

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

The fact that no issues are observed in safety studies on different enzymes (e.g. amylases, protease etc.) produced by strains derived from a common predecessor (*B. licheniformis* MDT233), strongly supports the safety of the *B. licheniformis* strain lineage, independent of which enzyme is produced.

The production strain is genetically modified by rDNA techniques as discussed in Part 2. The expressed xylanase enzyme preparation is free of DNA encoding transferable antibiotic resistance gene DNA. The introduced DNA is well characterized and safe for the construction of microorganisms to be used in the production of food grade products. The DNA is stably integrated into the chromosome and the incorporated DNA is known not to encode or express any harmful or toxic substances. The procedures used to modify the host organism are well defined and commonly used. Therefore, the elements needed to establish a safe strain lineage as defined in Pariza and Johnson, 2001 (19) have been met.

Based on the information presented in Parts 6 (a) and (b), it is concluded that the *Bacillus licheniformis* production strain is part of the safe strain lineage and is considered a safe strain for the production of the xylanase enzyme.

6(e) Safety of the Donor Organism

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

The donor organism of the xylanase is *chryseobacterium sp-10696*. As indicated in Part 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.

6(f) Safety of the Xylanase Enzyme

As indicated in Part 2, 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 (4) (22) 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 (4) (22) . Pariza and Foster (22) note that very few toxic agents have enzymatic properties. Also, the safety of the xylanase was assessed using the Pariza and Johnson, (2001) decision tree.

Also, A literature search was performed in January 2022 for the period of 2020 to current (January 13, 2022) on xylanase utilizing the database SCOPUS and key words “xylanase enzyme”, “safety”, “toxins”, “food” and derivatives of those words. A total of 50 relevant hits were found. Novozymes reviewed the totality of the available abstracts and found none to be inconsistent with our conclusion of the general recognition of safety of xylanase enzyme.

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.

6(g) Allergenic/Toxigenic 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.

More than 35% identity in the amino acid sequence of the expressed protein using a window of 80 amino acids as criterion, Phl p 4 was found showing up to 35.2% sequence identity. The identities across the full length was 9.6% which is low and thus not considered problematic.

Phl p 4 is a grass pollen allergen from timothy grass (*Phleum pretense*). Phl p 4 is glycosylated protein with cross-reactive carbohydrate determinants (CCDs) that has a low allergenicity because sensitized individuals develop IgE antibodies against the poorly antigenic CCDs (32).

No hits to any food allergens were found with high sequence identity to Xylanase produced by *Bacillus licheniformis* strain xylCB-0087, and no information is available on oral sensitization or elicitation reactions of Xylanase produced by *Bacillus licheniformis* strain xylCB-0087.

Similar to respiratory pollen allergy, the sensitizing potential of enzymes after respiratory exposure, in an occupational setting, and the relation to enzyme specific asthma, has been recognized for many years (33). This requires that Xylanase produced by *Bacillus licheniformis* strain xylCB-0087 must be handled according to established safety guidelines for enzymes.

No hits to any food allergens were found with high sequence identity to Xylanase produced by *Bacillus licheniformis* strain xylCB-0087, and no information is available on oral sensitization or elicitation reactions of Xylanase produced by *Bacillus licheniformis* strain xylCB-0087.

However, in general, there is compelling evidence that the majority of adults affected by occupational asthma to a food enzyme can ingest respiratory allergens without acquiring clinical symptoms of food allergy suggesting that inhalation is not likely to result in food allergy (34) (35) (36).

In addition, the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) Working Group on Consumer Allergy Risk from Enzyme Residues in Food performed an in-depth analysis of the allergenicity of enzyme products (37). In this paper, Dauvrin et al (1998) conclude that enzyme exposure by ingestion, in opposition to exposure by inhalation, is extremely unlikely to lead to sensitization.

This is backed up by a study using the generally recognized guidelines for food allergy diagnosis (skin prick test, specific serum IgE and DBPCFC). This study

included 400 patients with diagnosed allergy to one or more of inhalation allergens, food allergens, bee or wasp allergens. The study concluded that no cases of IgE-mediated food allergy to commercial enzymes could be found. There were further no indications of cross-reactivity between the tested enzymes used in food and the main known allergens represented by the patients included in the study. It was concluded from this study that ingestion of food enzymes in general is not likely to be a concern with regard to food allergy (29).

On the basis of the available evidence, it is concluded that oral intake of xylanase produced by *Bacillus licheniformis* xylCB-0087 is not anticipated to pose any food allergenic or toxigenic concerns.

6(h) Safety Studies

Novozymes has repeatedly used the procedures outlined by Pariza and Johnson to evaluate enzymes derived from *Bacillus licheniformis* production strains (19). As described in Part 6(c), Novozymes has concluded, that strains within the safe strain lineage of *Bacillus licheniformis* pose no safety concerns. Table 4 lists the strains within this lineage, with many having corresponding GRNs on file with the FDA, where toxicological safety studies have been performed.

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

It is reasonable to expect and conclude that enzymes produced by *Bacillus licheniformis* strains within this safe strain lineage will show similar toxicological profiles and further supports our conclusion that *Bacillus licheniformis* strains are safe hosts for the expression of enzymes (38) (39).

6(i) Description of the Test Article

Novozymes considers the xylanase enzyme preparation, produced by the *Bacillus licheniformis* production organism, to be safe. This was determined through scientific procedure and is based on a review of the toxicological studies conducted on strains within Novozymes' *Bacillus licheniformis* safe strain lineage.

The results from those studies indicate that the enzyme concentrates did not exhibit any toxic or mutagenic effects under the conditions of the test. Additionally, Novozymes conducted the following safety studies on the xylanase enzyme, subject of this notification, concentrate batch PPQ69045

- AMES (bacterial reverse mutation assay)
- *In vitro* micronucleus study
- Combined *In vivo* micronucleus test and Comet assay
- 13-week Oral Gavage in Rats

All toxicology studies performed on the xylanase enzyme concentrate 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 Labcorp (U.K.) and Helix3 (U.S.) during the period June 2020 to October 2021.

A more detailed summary of the toxicology studies for the xylanase enzyme concentrate batch PPQ69045 produced by *Bacillus licheniformis*, is included as an Appendix.

Based on the presented toxicity data, the history of safe use of the enzyme and the safe strain lineage of the *Bacillus licheniformis* production strain, it can be concluded that the test preparation; enzyme concentrate batch PPQ69045, exhibits no toxicological effects under the experimental conditions described in the summary.

6(j) 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 *Bacillus licheniformis* and the limited and well-defined nature of the genetic modifications, 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, 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

APPENDIX

Summary of Toxicity Data. Xylanase, batch PPQ69045 from *Bacillus licheniformis.* November 1, 2021. LUNA No. 2021-17107-02.

REFERENCES

1. NIH Guidelines: Guidelines for Research Involving Recombinant DNA Molecules. Federal Register (66 FR 57970). December 19, 2001.

2. Organisation for Economic Cooperation and Development, Safety Evaluation of Foods Derived by Modern Biotechnology, 1993.

3. Pariza, M.W. and Foster, E.M., Determining the Safety of Enzymes Used in Food Processing. Journal of Food Protection, 46:5:453-468, 1983.

4. 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.

5. Olempska-Beer, Z.S. , Merker, R.I, Ditto, Mary D., and DiNovi, M.J. Food-processing enzymes from recombinant microorganisms—a review. Reg. Tox and Pharm 45:144-158, 2006.

6. Nevalainen, Helena; Suominen, Pirkko; Taimisto Kaarina. On the safety of Trichoderma reesei, Journal of Biotechnology, Volume 37, June 1994.

7. Blumenthal CZ. Production of toxic metabolites in Aspergillus niger, Aspergillus oryzae, and Trichoderma reesei: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. Reg. Tox. and Pharma., 39:214-218, 2004.

8. Horinouchi, S., and Weisblum, B.. Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide and streptogramin type-b antibiotics. J. Bacteriol., 150, 804-814, 1982.

9. Gryczan, T.J., Contente, S., Dubnau, D. : Characterization of Staphylococcus aureus plasmids introduced by transformation into Bacillus subtilis. J. Bact. 134, 318-329 (1978).

10. IFBC (International Food Biotechnology Council). Chapter 4: Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms in Biotechnologies and Food: Assuring the Safety of Foods Produced by Genetic Modification. 1990, Vol. 12, pp. S1-S196.

11. Aunstrup, K., Andersen, O., Falch, E.A., and Nielsen, T.K.. Production of Microbial Enzymes in Microbial Technology, 2nd ed., Vol. I, Eds. Peppler, H.J. and Perlman, D., Chapter 9, pp.282-309, 1979.

12. Aunstrup, K.. Production, Isolation, and Economics of Extracellular Enzymes in Applied Biochemistry and Bioengineering, Volume 2, Enzyme Technology, Eds. Wingard, L.B., Katchalski-Katzir, E. And Goldstein, L, pp. 28-68, 1979.

13. Kirk, O., Damhus, T., Borchert, T. V., Fuglsang, C. C., Olsen, H. S., Hansen, T. T., et al. Enzyme Applications, Industrial. Kirk-Othmer Encyclopedia of Chemical Technology, Vol. 9, pp 566-620, 2000.

14. United States Pharmacopeial Convention. Food Chemical Codex. Edition 9. Monograph: Enzyme Preparations. United States Pharmacopeial Convention, Board of Trustees, 2014. Pg 410-415.

15. JECFA (Joint FAO/WHO Expert Committee on Food Additives. General Specifications and Considerations for Enzyme Preparations Used in Food Processing. Compendium of Food Additive Specifications, FAO FNP (Food and Nutrition Paper) 52, Add. 9, FAO, Rome 2001 and FAO JECFA Monographs 3 (2006).

16. Hansen, S.C. Acceptable Daily Intake of Food Additives and Ceiling on Levels of Use. Fd. Cosmet. Toxicol. 4, 427-432, 1966.

17. http://www.cdc.gov/nchs/nhanes.htm.

18. Bathgate, G.N. and Bringhurst, T.A. (2011) Letter to the Editor: Update on knowledge regarding starch structure and degradation by malt enzymes, J. Inst. Brew. 117, 33-38.

19. 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.

20. Sewalt Vincent, Shanahan Diane, Gregg Lori, La Marta James and Carrillo Roberts; The Generally Recognized as Safe (GRAS) Process for Industrial Microbial Enzymes. Industrial Biotechnology, Vol. 12, No. 5. October 2016.

21. United States Pharmacopeial Convention. Food Chemical Codex. Edition 10. Monograph: Enzyme Preparations. United States Pharmacopeial Convention, Board of Trustees, 2016. Pg 445-450.

22. Pariza, M.W. and Foster, E.M.. Determining the Safety of Enzymes Used in Food Processing. Journal of Food Protection, 46:5:453-468, 1983.

23. IFBC (International Food Biotechnology Council). Chapter 4: Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms in Biotechnologies and Food: Assuring the Safety of Foods Produced by Genetic Modification. 1990, Vol. 12, pp. S1-S196.

24. de Boer AS, Diderichsen B, On the safety of Bacillus subtilis and B. amyloliquefaciens: a review. Appl. Microbiol. Biotechnol, 1991.

25. GRAS Notice Inventory: http://www.accessdata.fda.gov.

26. EFSA, 2010, Scientific Opinion on the maintenance of the list of QPS microorganisms intentionally added to food and feed (2010 update). EFSA Journal 2010; 8(12):1944.

27. Pariza, M.W. and Cook, M. Determining the safety of enzymes used in animal feed. Reg. Tox and Pharm 56:332-342, 2010.

28. Beg QK., Kapoor M., Mahajan L., Hoondal GS. Microbial xylanases and their industrial applications: A review. Appl. Microbiology Biotechnology. 56, 326-338. 2001.

29. Bindslev-Jensen C, Skov PS, Roggen EL, Hvass P, Brinch DS Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry. Food Chem. Toxicol. 44, 1909-1915, 2006.

30. Evaluation of Allergenicity of Genetically Modified Foods (Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology 22 25 January 2001), Food and Agriculture Organization of the United Nations (FAO), Rome, 2001.

31. Codex Alimentarius commission. Foods derived from modern biotechnology. FAO/WHO, Rome, pp.1-85, 2009.

32. Mendy, A., Zeldin, DC., 2020. J Allergy Clin Immunol. 145(6):1556-1557. June 2020. .

33. Sarlo K., Ann Allergy Asthma Immunol. 90(5 Suppl 2):32-4. May 2003.

34. Cullinan P, Cook A, Jones M, Cannon J, Fitzgerald B and Newman Taylor AJ. Allergy, 52, 346–349, 1997.

35. Armentia, A., Díaz_Perales, A., Castrodeza, J., Dueñas-Laita, A., Palacin, A., Fernández S. Allergol. Immunopathol. 37; 203-204, 2009.

36. Poulsen LK. Molecular Nutrition & Food Research, 48, 413–423, 2004. .

37. Dauvrin T, Groot G, Maurer K-H, de Rijke D, Ryssov-Nielson H, Simonsen M, Sorensen TB., Working group on consumer allergy risk from enzyme residues in food. Amfep, 1998.

38. EFSA Panel on Genetically Modified Organisms (GMO); Scientific Opinion on Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use. EFSA Journal 2011;9(6):2193 [54 pp] joi:10.2903/j.efsa.2011.

39. Van Dijck et. al, On the safety of a new generation of DSM Aspergillus niger enzyme production strains. Regulatory Tox. and Pharma, 38:27-35. 2003.

Toxicology & Immunology

Date: November 01, 2021
File: 2021-17107-02
Ref.: BTR

SUMMARY OF TOXICITY DATA

Xylanase, batch PPQ69045 from *Bacillus licheniformis*

Author:
Birgitte Thue Ravn
Trine Ø. Lund

Issued by:
Novozymes A/S
Krogshoejvej 36
DK-2880 Bagsvaerd
Denmark

CONTENTS

PAGE

1. ABSTRACT

The below series of toxicological studies were undertaken to evaluate the safety of Xylanase, represented by batch PPQ69045.

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 Labcorp (UK) and Helix3 (US) during the period June 2020 to October 2021.

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

- Xylanase, batch PPQ69045 was not genotoxic *in vivo*.
- In a 13-week oral toxicity study in rats Xylanse, batch PPQ69045 was well tolerated and did not cause any toxicologically significant changes at any dose level applied.

Based on the present toxicity data, it can be concluded that Xylanase, represented by batch PPQ69045, exhibits no significant toxicological effects under the experimental conditions described.

2. TEST SUBSTANCE

2.1 Characterization

The toxbatch Xylanase, batch PPQ69045, was used for the conduct of all the toxicological studies. The characterization data of the toxbatch is presented in Table 1.

Table 1. Characterization data of Xylanase, batch PPQ69045

Batch number	PPQ69045
Activity	330 FXU(TB)/g
N-Total (% w/w)	0.86
Water (KF) (% w/w)	89.0
Dry matter (% w/w)	11.0
Ash (% w/w)	1.7
Total Organic Solids (TOS[1]) (% w/w)	9.3
Density (g/mL)	1.034

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

3. MUTAGENICITY

3.1 Review of Genotoxicity Data

965155evaluation of data on genotoxic potential is recommended (EFSA 2009, EFSA 2011).

Xylanase, batch PPQ69045 was initially assessed in a battery of *in vitro* genotoxicity studies. The bacterial reverse mutation assay (Ames test) showed clearly negative results both in the absence and presence of metabolic activation (S-9). In the *in vitro* micronucleus test, Xylanase, batch PPQ69045 induced increases in micronuclei in cultured human peripheral blood lymphocytes following treatment in the absence and presence of S-9. Subsequent mechanistic analysis via the use of fluorescence *in* situ hybrisation (FISH) with pan-centromeric DNA probes clearly demonstrated that micronuclei were generated via a predominantly clastogenic (chromosome breakage) mechanism.

According to the EFSA guidance, further assessment is needed in case a positive result is obtained *in vitro* (EFSA 2009, EFSA 2011). Conducting an *in vivo* study on Xylanase, batch PPQ69045 was considered necessary to assess whether the genotoxic potential observed *in vitro* was expressed *in vivo*. *In vivo* tests should relate to the genotoxic endpoint(s) identified as positive *in vitro* and to appropriate target organs or tissue. Evidence that the target tissue(s) have been exposed to the test substance and/or its metabolites is essential for interpretation of negative results. Combination studies (assessing different endpoints in different tissues in the same animal) should be considered if there is no kinetic evidence that the agent reaches the tissue under investigation (EFSA 2011).

Xylanase, batch PPQ69045 was negative in the Ames test and positive in the *in vitro* micronucleus test (with demonstration of a clastogenic mechanism). Due to the nature of this test substance, demonstration of exposure to the bone marrow would not be possible. Based on this, an oral gavage *in vivo* combined micronucleus test and comet assay, including first-site-of-contact tissue (stomach and duodenum) and liver tissue, was considered an appropriate follow-up study design.

In the conducted *in vivo* combined micronucleus test and comet assay, Xylanase, batch PPQ69045 did not show any evidence of causing an increase in the induction of micronucleated polychromatic erythrocytes or bone marrow cell toxicity in male Crl:CD(SD) rats when administered orally by gavage. It was furthermore concluded that Xylanase, batch PPQ69045 had not shown any evidence of causing an increase in DNA strand breaks in the liver of the rats, whereas equivocal results were obtained in the duodenum and glandular stomach due to increases in comet tail intensities observed in combination with the confounding presence of hedgehog cells.

In an expert review performed on the comet part of the study, several technical issues, which could potentially lead to artifactual dose related trends, were identified and the comet data generated were considered questionable and potentially unreliable. Based on this, the comet part of the study was repeated with the same experimental design but with technical adjustments according to the recommendations given in the expert review, to obtain clarification and an overall conclusion based on data with improved reliability. In the repeated comet assay, Xylanase, batch PPQ69045 was clearly negative for inducing genotoxicity in the liver, duodenum, and glandular stomach of treated animals up to the maximum feasible dose of 1923.24 mg TOS/kg. These results were obtained in a study adjusted based on a scientifically highly well-founded knowledge and are therefore

considered strong, reliable, and compelling. Hence, the study conclusion is regarded as suitable for an overall clarification of the genotoxicity assessment.

Based on the results of the battery of appropriate *in vitro* and *in vivo* tests conducted on Xylanase, batch PPQ69045, the overall conclusion is that the genotoxic potential observed in the *in vitro* micronucleus test was not relevant for the *in vivo* situation. Hence, Xylanase, batch PPQ69045 is not genotoxic *in vivo.*

For a detailed summary and discussion of the genotoxicological studies, please refer to 'Review of Genotoxicity Data on Xylanase batch PPQ69045'.

4. GENERAL TOXICITY

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

The purpose of this study was to assess the systemic toxic potential of Xylanase, batch PPQ69045 (an enzyme used in the food industry) after once daily oral, by gavage, administration to Han Wistar rats for 13 weeks.

Three groups, each comprising ten male and ten female RccHan™;WIST rats, received 10, 33 or 100 % of Xylanase, batch PPQ69045 (equivalent to 96.2, 317 or 962 mg TOS/kg bwt/day or 341.2, 1126 or 3412 FXU(TB)/kg bwt/day, respectively) at a dose volume of 10 mL/kg bwt/day. A similarly constituted control group received the vehicle (reverse osmosis water) at the same volume dose as treated groups.

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

The general appearance and behaviour of the animals and sensory activity, grip strength and motor activity were unaffected by treatment. Females receiving 100 % of Xylanase, batch PPQ69045 had a slight (9.7%) reduction of overall body weight gain, however, this was considered not to be related to treatment but to represent normal biological variation. There was no effect of treatment on food and water consumption.

There were no treatment-related ophthalmoscopic findings.

The haematological and blood chemistry investigations did not indicate any toxicologically significant findings. In the haematological examination there was a small increase in activated partial thromboplastin time in males receiving 100% of Xylanase, batch PPQ69045 and on the contrary, a decrease in prothrombin times was observed at all doses in males and in females receiving 100% of Xylanase, batch PPQ69045, however, the observed differences were considered incidental and of no toxicological importance. In the blood plasma there was an increase of urea/blood urea nitrogen concentration in males receiving 100 % of Xylanase, batch PPQ69045 and low bile acid concentration in females receiving 33 or 100 % of Xylanase, batch PPQ69045, however, the observed differences were considered to represent normal biological variation.

Estrous cycles at the end of the treatment period were unaffected. There was also no effect on testicular pathology.

Serum triiodothyronine (T3), thyroxine (T4) concentrations were considered to have been unaffected by treatment. Thyroid stimulating hormone (TSH) levels were statistically significantly low in females given 100% Xylanase, batch PPQ69045, however, the observed difference was considered to represent normal biological variation.

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

It is concluded that oral administration of Xylanase, batch PPQ69045 at a dose volume of 10 mL/kg bwt/day to Han Wistar rats at doses up to 100% of the test batch (equivalent to 962 mg TOS/kg bwt/day or 3412 FXU(TB)/kg bwt/day) for 13 weeks was well-tolerated, with no evidence of any adverse finding at any of the administered doses. Consequently, the no-observed-adverse-effect level (NOAEL) was considered to be 962 mg TOS/kg bwt/day (equivalent to 3412 FXU(TB)/kg bwt/day).

5. REFERENCES

5.1 Study reports and Reviews

Review of Genotoxicity Data on Xylanase batch PPQ69045 (October 2021). LUNA file: 2021-16725.

Covance: Study No.: 8430528; Novozymes Reference No.: 20206008: Xylanase, Batch PPQ69045: Toxicity Study by Oral Gavage Administration to Han Wistar Rats for 13 Weeks. (March 2021). LUNA file: 2021-06000.

5.2 Publications and Guidance Documents

EFSA 2009. Guidance of the Scientific Panel of Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) on the Submission of a Dossier on Food Enzymes for Safety Evaluation by the Scientific Panel of Food Contact Material, Enzymes, Flavourings and Processing Aids (EFSA Journal (2009) 1305, 1-26).

EFSA 2011. European Food Safety Authority, Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment (EFSA Journal 2011;9(9):2379).

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
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	Sr. Regulatory Affairs Specialist

Company *(if applicable)*: Novozymes North America

Mailing Address *(number and street)*: 77 Perrys Chapel Church Road, P.O. Box 576

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

Telephone Number	Fax Number	E-Mail Address
919-494-8784		jao@novozymes.com

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

Name of Contact Person	Position

Company *(if applicable)*:

Mailing Address *(number and street)*:

City	State or Province	Zip Code/Postal Code	Country

Telephone Number	Fax Number	E-Mail Address

PART III – GENERAL ADMINISTRATIVE INFORMATION

1. Name of Substance

Xylanase from Bacillus licheniformis

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 xylanase enzyme preparation is used as a processing aid during food manufacturing to aid in the separation of grains into the germ, starch, gluten and fiber. The enzyme can be used in any food application where the starch that is present can be modified by the xylanase. Some examples of these applications include starch processing, brewing and other cereal based beverage processes. 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

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

☐ Yes ☒ No

PART V – IDENTITY

1. Information about the Identity of the Substance

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

The substance is a xylanase. IUBMB nomenclautre: endo-1,4-beta-xylanase. Specificity is: hydrolyzes internal glucosidic beta-1,4 bonds in arabinoxylan. kDA is 60.8. The typical composition of the enzyme preparation is: water, enzyme, sodium benzoate and potassium sorbate.

3. Synonyms

Provide as available or relevant:

1	
2	
3	

PART VI – OTHER ELEMENTS IN YOUR GRAS NOTICE

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

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

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☒ Yes ☐ No

Did you include this other information in the list of attachments?

☒ Yes ☐ No

PART VII – SIGNATURE

1. The undersigned is informing FDA that Novozymes North America *(name of notifier)*

has concluded that the intended use*(s)* of Xylanase from Bacillus licheniformis *(name of notified substance)*

described on this form, as discussed in the attached notice, is (are) exempt from the premarket approval requirements of section 409 of the Federal Food, Drug, and Cosmetic Act because the intended use(s) is (are) generally recognized as safe.

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

Novozymes North America *(name of notifier)* agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so.

(address of notifier or other location)

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

OR

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

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
Janet Oesterling Digitally signed by Janet Oesterling Date: 2022.01.13 13:19:36 -05'00'	Janet Oesterling, Sr. Regulatory Affairs Specialist	01/13,2022

PART VIII – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	GRASNotification_Xylanase from Bacillus licheniformis_2022-01-13.pdf	Submission
	SummaryofToxicityData_Xylanase from Bacillus licheniformis_2021-11-21.pdf	Administrative

Novozymes
GRN 1055- Response to FDA Questions
February 2023

1. Please clarify the species of *Chryseobacterium* that is the source of the xylanase gene.

   ANSWER: The source of the xylanase gene is Chryseobacterium cucumeris (KACC 18798). This was determined using the Genome Taxonomy DataBase (GDTB) (Parks DH et. al 2018 and Chaumeil PA et al 2018) where the entire genome sequence is used for identification, rather than the unique 16S rRNA sequence. The result identifies the donor strain as Chryseobacterium cucumeris (KACC 18798). Chryseobacterium cucumeris belongs to risk group class 1.

2. For the administrative record, please state the number of amino acids in the xylanase.

   ANSWER: 551

3. Please state whether *B. lichenformis* strain SJ14481 has been deposited in a recognized culture collection.

   ANSWER: Novozymes confirms that the B. licheniformis strain SJ14481 has been deposited in a recognized culture collection.

4. On pages 6 and 7 of the notice, the notifier states "The recipient strain used in the construction of the xylanase production strain was modified at several chromosomal loci during strain development to inactivate genes encoding a number of proteases. Also, deletion of a gene essential for sporulation was performed, eliminating the ability to sporulate, together with the deletion of additional genes encoding unwanted proteins that can be present in the culture supernatant."

   For the administrative record, please clarify and confirm that any modifications to the native genes, especially those termed "unwanted proteins", are not expected to alter the safety profile of the production strain.

   ANSWER: The production strain is derived from a safe strain lineage, for which we have data confirming the safety of the modifications. Furthermore, strains from this safe strain lineage have been approved and used in food and feed products for several years. When referring to "unwanted proteins" in our description of the strain, we primarily think of proteins which can make the purification and recovery process of the enzyme more difficult.

5. The notifier states on page 7 of the notice that the expression cassette contains a sequence "encoding the modified xylanase." Please clarify and confirm that any modifications to the xylanase gene are not expected to significantly alter the activity and/ or stability of the enzyme such that that safety profile of the enzyme would be affected.

   ANSWER: The xylanase gene originating from the risk group 1 organism Chryseobacterium cucumeris (KACC 18798), has only been modified slightly to obtain our production xylanase gene, xylCB-0087. The modified xylanase gene is >98% identical to the mature amino acid sequence of the wild type xylanase. Due to the high similarity between the native xylanase and the modified xylanase, the changes are not expected to change the safety profile of the enzyme.

6. Please state whether the enzyme is secreted or lysed.

   ANSWER: Secreted

7. Please indicate the color of the liquid enzyme concentrate.

   ANSWER: Tan to brown

8. Please list the methods used to analyze the batches for conformance with the stated specifications and indicate if they have been validated for that particular purpose.

   ANSWER: The methods of analysis used to determine the analytical data are validated methods. Some are in-house methods evaluated and validated at Novozymes and are indicated as such in the Table below.

Xylanase activity unit	Novozymes internal method
Lead	DTI (Danish Technologial Institute) UA-255
Total Coliforms	ISO 4832
Salmonella	Novozymes internal method
Escherichia coli	ISO 16649-2 and ISO 16654
Antimicrobial activity	In accordance with FAO/JECFA Monographs (2006), pages 122-123

9. The specification for lead is 5 mg/kg. Please consider reducing the specification for lead to reflect the results from the batch analyses.

   ANSWER: Noted

10. In Table 1, the TOS is estimated as 7.4% of the enzyme preparation. On page 12, the enzyme activity/TOS ratio is calculated using 6.4% TOS. Please clarify and update the dietary exposure calculations.

    ANSWER: The average TOS is 6.4%. The TOS number indicated in Table 1 was a typo. The calculation for exposure in the notification is correct and is based on 6.4% TOS.

11. On page 13, the use level for brewing and cereal based beverage applications is calculated as 80 mg TOS/ kg starch. Further on page 14, for the same use, 68.5 mg TOS/ kg starch is used. Please clarify this discrepancy.

    ANSWER: 68.5 is the correct mg TOS/kg starch in the Brewing and Cereal Based Beverage application. The TMDI calculated for this application using 68.5 is correct. The corresponding Safety Margin indicated in the notification is calculated correctly. Please disregard the 80 mg TOS/kg starch number.

References

Parks DH, et al. 2018. A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life. Nat. Biotechnol., http://dx.doi.org/10.1038/nbt.4229

Chaumeil PA, Hugenholtz P, Parks DH. 2018. GTDB-Tk: A toolkit to classify genomes with the Genome Taxonomy Database. https://pubmed.ncbi.nlm.nih.gov/31730192/

Overbey, Katie

From: JAO (Janet Oesterling) <JAO@novozymes.com>
Sent: Tuesday, March 7, 2023 8:47 AM
To: Overbey, Katie
Subject: [EXTERNAL] Novozymes response - Additional Question - GRN 1055

Hi Katie,

The enzyme in this sentence on page 14 is a typographical error. The enzyme should be xylanase NOT amylase.

Best regards,

Janet Oesterling
Regulatory Affairs Manager

Novozymes North America Inc.
PO BOX 576
77 Perrys Chapel Church Road
Franklinton NC 27525 USA
Phone: +1 9194943000
Mobile: +1 2529151444
E-mail: jao@novozymes.com

Find Novozymes on: Web | LinkedIn | Twitter | Facebook

From: Overbey, Katie <Katie.Overbey@fda.hhs.gov>
Sent: Friday, March 3, 2023 11:50 AM
To: JAO (Janet Oesterling) <JAO@novozymes.com>
Subject: Additional Question - GRN 1055

Hello Dr. Oesterling,
We had an additional clarifying question that came up in our review for GRN 1055:

1. On page 14, you state "The NOAEL dose level in the 13-week oral toxicity study in rats conducted on ***amylase*** tox batch PPQ69045 was the highest dosage possible, 962 mg TOS/ kg bw/day." Please clarify if the stated enzyme in this sentence is a typographical error and if the sentence is intended to state "***xylanse*** tox batch."

Please format your response so that your answer immediately follows the questions. We request that you provide a response within 10 business days.

Let me know if you have any additional questions or need any clarification.

Best,
Katie

Katie Overbey, Ph.D., M.S (she/her/hers)
Regulatory Review Scientist

Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
Tel: 240-402-7536
katie.overbey@fda.hhs.gov