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
GRN 000751	Dec 22, 2017
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

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

PART I – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

☒ New ☐ Amendment to GRN No. _____ ☐ Supplement to GRN No. _____

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

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

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

PART II – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position
Janet Oesterling	Regulatory Affairs

Company *(if applicable)*: Novozymes North America

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

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

Maltogenic alpha-amylase from Bacillus stearothermophilus produced by Bacillus subtilis

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 maltogenic alpha-amylase preparation is used as a processing aid during food manufacturing. The enzyme can be used in any food application where the starch present can be modified by the maltogenic alpha-amylase. Some examples of these applications include starch processing, baking and other cereal based processes, brewing processes and other cereal based beverage processes. The dosage applied in practice by a food manufacturer depends on the process and the initial recommendation by the enzyme manufacturer. The dose is optimised to fit the process conditions. There is no special population for which this enzyme preparation is intended.

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	maltogenic alpha amylase	EC	3.2.1.133		
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 enzyme is a maltogenic alpha amylase from Bacillus stearothermophilus produced by bacillus subtilis. The specificity catalyzes the hydrolysis of 1,4-alpha-glylcosidic linkages in amylose, amylopectin, and related glucose polymers. And, was produced by pure culture submerged fed-batch fermentation.

The quantitative composition of the enzyme preparation consists of: enzyme solids, wheat flour, water, sodium chloride, sucrose, glycerol, sodium benzoate and potassium sorbate.

3. Synonyms

Provide as available or relevant:

1	4-alpha-D-glucan alpha-maltohydrolase
2	glucan 1,4-alpha-maltohydrolase
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 Maltogenic alpha-amylase from Bacillus stearothermophilus produced by Bacillus subtilis *(name of notified substance)*

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

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

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

77 Perrys Chapel Church Rd, Franklinton, NC 27525 *(address of notifier or other location)*

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

OR

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

3. Signature of Responsible Official, Agent, or Attorney	**Printed Name and Title**	**Date (mm/dd/yyyy)**
janet oesterling Digitally signed by janet oesterling Date: 2017.12.19 11:05:17 -05'00'	Janet Oesterling, Regulatory Affairs	12/19/2017

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_Maltogenicalpha-amylase_ 2017-12-18.pdf	Submission
	DecisionTree_Maltogenic alpha-amylase_2017-12-18.pdf	Administrative
	Part 1_Maltogenic alpha-amylase_2017-12-18.pdf	Administrative
	SummaryofToxicityData_Maltogenic alpha-amylase_2002-02.pdf	Administrative
	Sewalt etal_GRAS Process for Industrial Microbial enzymes.pdf	Administrative

OMB Statement: Public reporting burden for this collection of information is estimated to average 150 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services,Food and Drug Administration, Office of Chief Information Officer, 1350 Piccard Drive, Room 400, Rockville, MD 20850. (Please do NOT return the form to this address.). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.

PART 1: Signed statement of the conclusion of GRAS (Generally Recognized as Safe) and certification of conformity to 21 CFR §170.205-170.260.

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

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

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

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

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

The appropriately descriptive term for this notified substance is: Maltogenic alpha-amylase enzyme from *Bacillus stearothermophilus* produced by *Bacillus subtilis.*

§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 maltogenic alpha-amylase enzyme will be used as a processing aid during food manufacturing. The enzyme preparation will be used in a variety of applications such as; starch processing, baking and other cereal based processes, brewing and other cereal based beverages. 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.

Maltogenic alpha-amylase from Bacillus stearothermophilus produced by Bacillus subtilis. LUNA #2017-19245-01.

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

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

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

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

(b) (6)

12/18/17

Janet Oesterling
Regulatory Affairs Specialist III

Date

A Maltogenic Alpha-Amylase from *Bacillus stearothermophilus* Produced by *Bacillus subtilis*

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

December 2017

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 maltogenic alpha-amylase produced by submerged fermentation of a genetically modified *Bacillus subtilis* microorganism expressing the gene encoding a maltogenic alpha-amylase from *Bacillus stearothermophilus.*

Key enzyme and protein chemical characteristics of the maltogenic alpha-amylase are given below:

Systemic Name:	4-alpha-D-glucan alpha-maltohydrolase
Accepted Name:	glucan 1,4-alpha-maltohydrolase
EC No.:	3.2.1.133
CAS No.:	160611-47-2
Specificity:	catalyzes the hydrolysis of 1,4-alpha-glylcosidic linkages in amylose, amylopectin, and related glucose polymers.
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 subtillis* production strain, designated BRG-1, was derived from parental strain A164 via the recipient strain A164Δ5. Strain A164 is identical to strain ATCC 6051a, the wild type strain of *Bacillus subtilis*. This genetically modified production organism complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms (1). It also meets the criteria for a safe production microorganism as described by Pariza and Foster (2) and later Pariza and Johnson (3) and several expert groups (4) (5) (1) (6) (7) (8).

The *amyM* expression plasmid (pNBT24-*amyM)*, used in the strain construction, contains strictly defined chromosomal DNA fragments and synthetic DNA linker sequences. The DNA sequence for the introduced gene is based on the *amyM* coding sequence from *Bacillus stearothermophilus*. In the *amyM* expression cassette used for the construction of the production strain, the DNA sequence encoding the maltogenic alpha-amylase includes the native signal peptide from the *amyM* gene of *Bacillus stearothermophilus*, to enable efficient secretion.

2.2(b) Recipient Strain

The recipient strain A164Δ5 used in the construction of the *amyM* 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) Maltogenic Alpha-Amylase Expression Plasmid

The expression plasmid, pNBT24-*amyM*, used to transform the *Bacillus subtilis* recipient strain A164Δ5 is based on the well-known *E. coli* 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 the *Bacillus amyloliquefaciens* and *Bacillus thuringiensis* engineered promoter, the *amyM* coding sequence and a transcriptional terminator.

Following the terminator, a non-coding DNA sequence is inserted to enable targeted integration of the transforming DNA into the genome of the recipient strain. Only the expression cassette with elements between the promoter fragment and the terminator are present in the final production strain. This has been confirmed by Southern blot analysis and PCR analysis followed by DNA sequencing.

2.2(d) Construction of the Recombinant Microorganism

The production strain, *Bacillus subtilis* BRG-1, was constructed from the recipient strain A164Δ5 through the following steps:

1) Plasmid pNBT24-amyM was integrated into a single specific locus (amyE) in strain A164Δ5 by targeted homologous recombination to this locus using a two-step integration approach. Targeted integration of the expression cassette at this locus allows the expression of the amyM gene from the promoter. The resulting strain containing a single copy of the amyM gene at the target locus was named RB88.

2) Plasmid pRB115 was used to remove the chloramphenicol resistance marker by targeted homologous recombination. The targeted deletion of the chloramphenicol resistance gene was confirmed by selection of the chloramphenicol sensitive strain RB128.

3) The production strain BRG-1 was obtained from RB128 by classical mutagenesis and screening to identify a strain with increased maltogenic alpha-amylase activity.

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

2.2(e) Stability of the Introduced Genetic Sequences

The genetic stability of the introduced DNA sequences was determined by Southern hybridization. Analysis of samples from end of production using an *amyM* gene specific probe showed an identical band pattern compared to the reference production strain (BRG-1), demonstrating the genetic stability of the introduced DNA during production. The transforming DNA is stably integrated into the *Bacillus subtilis* chromosome and, as such, is poorly mobilized for genetic transfer to other organisms and is mitotically stable.

2.2(f) Antibiotic Resistance Gene

The *Bacillus subtilis* BRG-1 production strain was constructed from the *Bacillus subtilis* host strain A164Δ5 by chromosomally integrating a DNA fragment containing well-defined DNA sequences originating from *Bacillus amyloliquefaciens, Bacillus thuringiensis, Bacillus stearothermophilus* and *Bacillus subtilis.*

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 Southern hybridization.

2.2(g) Absence of Production Organism in Product

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

2.3 METHOD OF MANUFACTURE

This section describes the manufacturing process for the maltogenic alpha-amylase which follows standard industry practices (10) (11) (9). The quality management system used in the manufacturing process for the maltogenic alpha-amylase complies with the requirements of ISO 9001. It is produced under a standard manufacturing process as outlined by Aunstrup (11) 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 (12). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (13).

2.3(a) Raw Materials

The raw materials used in the fermentation and recovery process for the maltogenic alpha-amylase enzyme concentrate are standard ingredients used in the enzyme industry (9) (11) (10). 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 maltogenic alpha-amylase is produced by pure culture submerged fed-batch fermentation of a genetically modified strain of *Bacillus subtilis* as described in Part 2. During fermentation, the enzyme produced by *Bacillus subtilis* is secreted into the fermentation media. 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 subtilis,* 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

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) Preservation and Stabilization of the liquid enzyme concentrate

6) Final concentration – evaporation and/or ultrafiltration.

The liquid enzyme concentrate is stabilized with sucrose, glycerol and sodium chloride and preserved with potassium sorbate and sodium benzoate. After final concentration by evaporation and/or ultrafiltration, the concentrate is spray dried which results in a highly concentrated granulated product. The granulated product is further formulated by the addition of wheat flour. See Table 1 below.

2.4 COMPOSITION AND SPECIFICATIONS

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

2.4(a) Quantitative Composition

Table 1 below identifies the substances 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 composition raw materials of the enzyme preparations

Substance	Approximate Percentage
Enzyme Solids (TOS*)	4.3%
Wheat Flour	>90%
Water	45 – 50%
Sodium Chloride	5 – 75%
Sucrose	5 – 35%
Glycerol	5 – 35%
Sodium Benzoate	<0.5%
Potassium Sorbate	<0.5%

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

2.4(b) Specifications

The maltogenic alpha-amylase 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 (13).

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

Table 2. Analytical data for three food enzyme batches.

Parameter	Specifications	ABKA2361	ABKA2362	ABKA2363
Maltogenic Alpha-Amylase activity	MANU/g	19200	10300	13400
Total viable count	Upper limit 50,000	<100	2500	1400
Lead	Not more than 5 mg/kg	ND	ND	ND
Salmonella sp.	Absent in 25 g of sample	ND	ND	ND
Total coliforms	Not more than 30 per gr	< 4	< 4	< 4
Escherichia coli	Absent in 25 g of sample	ND	ND	ND
Antimicrobial activity	Not detected	ND	ND	ND
Production Organism	Not detected	ND	ND	ND

2.5 PHYSICAL OR TECHNICAL EFFECT

2.5(a) Mode of Action and Application

The active enzyme is a maltogenic alpha-amylase (EC 3.2.1.133). Maltogenic alpha-amylase catalyses and liberates maltose units from the non-reducing end of starch polymer chains by the catalyzed hydrolysis of 1,4-alpha-glucosidic linkages in amylose, amylopectin and related glucose polymers (hydrolysis-products of starch).

The main reaction product in the hydrolysis is maltose. Other starch derived hydrolysis-products, consisting of D-glucose units connected in chains of variable length, are also generated including single D-glucose units. These are all natural constituents of cereal-containing foods.

The maltogenic alpha-amylase preparation is used as a processing aid during food manufacturing. The enzyme can be used in any food application where the starch present can be modified by the maltogenic alpha-amylase. Some examples of these applications include starch processing, baking and other cereal based processes, brewing processes and other cereal based beverage processes.

Stabilization of the manufacturing process, less batch to batch variability, higher yields and flexibility of raw material choices are just a few of the benefits of using this enzyme.

2.5(b) Use Levels

The maltogenic alpha-amylase is not added to final foodstuffs, but is used as a processing aid during food manufacturing to hydrolyze starch.

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

The dosage applied in practice by a food manufacturer depends on the process and the initial recommendation by the enzyme manufacturer. The dose is optimised to fit the process conditions. The following are maximum suggested use levels for the listed food processing applications.

Starch processing

Up to 16500 MANU per kilogram of starch dry matter

Baking processes and other cereal based processes

Up to 15000 MANU per kilogram of starch dry matter.

Brewing processes and other cereal based beverage processes

Up to 16000 MANU per kilogram of starch dry matter.

2.5(c) Enzymes Residues in the Final Food

The maltogenic alpha-amylase food enzyme catalyses the hydrolysis of 1,4-alpha-glucosidic linkages in amylose, amylopectin and related glucose polymers (hydrolysis-products of starch). The effect of the maltogenic alpha-amylase is the conversion, during food processing, of starch in cereal-containing foods. This results in maltose and other starch derived hydrolysis-products consisting of D-glucose units of variable length.

In starch processing, the maltogenic alpha-amylase is typically added during saccharification (50-80°C, pH 3-7), where it degrades starch polysaccharides into maltose and glucose. The conditions in the saccharification step depend on the type of syrup that should be produced.

The production of all baked products includes mixing wheat flour or other cereal flour with other ingredients and water to prepare a dough or batter. This dough/batter is allowed to rest or ferment and is subsequently formed to the appropriate shape. The dough/batter is then either baked, boiled or steamed at high temperatures. The maltogenic alpha-amylase is added, together with other raw materials, during the dough formation step. The enzyme performs its function after starch gelatinisation (48-62°C) and until temperatures reach approximately 90°C (pH 4 9).

For cereal based processes, the maltogenic alpha-amylase is added to the cereal kernels before cooking to reduce the tendency of crystallisation of starch polymers.

When used in brewing or other cereal based beverage processes the maltogenic alpha-amylase is added at the beginning of the mashing, where it takes part in the degradation of starch into hydrolysis products of various chain. The mashing process takes from 1.5 to 5 hours (pH 5-6) and will often have a stepwise increase of the temperature, starting at 38-67°C up to 75-80°C depending of the type of beer, raw materials and enzymes.

The enzyme used during processing does not exert any unintentional enzymatic activity in the final food. This is due to 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 enzyme during processing, depletion of the substrate, lack of water activity, wrong pH, etc. In some cases, (e.g. products resulting from starch processing and distilling), the enzyme may no longer be present in the final food due to the processing steps applied during manufacturing of the final food.

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

PART 3 - DIETARY EXPOSURE

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

3(a) Assumptions in Dietary Exposure

The assumptions are highly exaggerated since the enzyme protein and the other compositional 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. Overall, the human

exposure to the maltogenic alpha-amylase 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.

The maximum recommended dosage used to calculate the dietary exposure is 16500 MANU per kg starch derived dry matter. This corresponds to 49.5mg TOS/kg bw/day.

3(b) Food Consumption Data

The exposure assessment for adults was performed using the Budget Method (15) (16) (17). The Budget Method assumptions represent a "maximum worst case" scenario of human consumption. The maltogenic alpha-amylase enzyme is assumed to be used at its maximum recommended dosages in the production of all processed beverages. It is also assumed that the totality of the maltogenic alpha-amylase enzyme will end up in the final food.

Assumptions in the Budget Method

The maltogenic alpha-amylase concentrate has an average activity of 14300 MANU/g and approximate 4.3% TOS (Total Organic Solids) content.

This corresponds to an activity/TOS ratio of 333 MANU/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 25g food. Therefore, adults ingest 25g food per kg body weight per day.

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

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

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

49.5mg TOS per kg/1000 x 3.12g = 0.154mg TOS

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 13% starch (or starch-derived) dry matter = 3.25g starch derived dry matter per kg bw per day.

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

Based on this, 3.25g starch derived dry matter in liquids will maximally contain:

49.5mg TOS per kg/1000 x 3.25g = 0.161mg TOS

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

0.154 + 0.161 = 0.315mg TOS/kg body weight/day

Theoretical Maximum Daily Intake (TMDI)

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

The NOAEL dose level in the 13-week oral toxicity study in rats conducted on maltogenic alpha-amylase tox batch PPY7087 was the highest dosage possible, 968.2mg TOS/kg bw/day. See Table 3 below.

Table 3. NOAEL Calculation

NOAEL (mg TOS/kg bw/day)	969
*TMDI (mg TOS/kg bw/day)	0.315
Safety margin	3076

*based on the worst-case scenario

PART 4 - SELF-LIMITING LEVELS OF USE

This part does not apply

PART 5 - COMMON USE IN FOOD BEFORE 1958

This part does not apply

PART 6 - NARRATIVE ON THE CONCLUSION OF GRAS STATUS

The information provided in the following sections is the basis for our determination of general recognition of safety of the maltogenic alpha-amylase enzyme preparation. Our safety evaluation in Part 6 includes an evaluation of the production organism, the donor strain, the introduced DNA, the enzyme and the manufacturing process. Data and information cited in this notification is generally available and Part 6 does not contain any data or information that is exempt from disclosure under the FOIA.

6(a) Safety of the Production Organism

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food (2) (3). The production organism for the maltogenic alpha-amylase, *Bacillus subtilis,* is discussed in Part 2 and in this Part.

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 (4). 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”.

Bacillus subtilis is a soil and plant living saprophyte, recognized as non-pathogenic and non-toxigenic species for humans, animals and plants (18). The microorganism is a common contaminant in foods eaten by humans and animals and it is consumed in large quantities when eating the Japanese food natto (19) (20).

Bacillus subtilis species have been used for centuries for production of natto by solid-state fermentation of soybeans. Industrial strains belonging to the *Bacillus subtilis* species have been used for decades in the production of enzymes, and more than a decade as recombinant organisms to produce a variety of bio-industrial products like food grade enzymes, vitamins, antibiotics, and additives (21).

Carbohydrase and proteases from *Bacillus subtilis* are affirmed as GRAS by the US FDA and are covered in the regulations under 21 CFR 184.1148 and 21 CFR 184.1150. Also, *Bacillus subtilis* is described as the production organism for different enzymes in GRAS notifications 20, 114, 205, 274, 406 and 476 (22). And, maltogenic alpha-amylase from *Bacillus subtilis* carrying the gene encoding maltogenic alpha-amylase from *B. stearothermophilis* is also in GRASP petition 7G0326 and is considered GRAS, as noted in FR 51, March 15, 1990.

According to the National Institutes of Health Guidelines for Research Involving Recombinant Molecules *Bacillus subtilis* is a class 1 organism and is generally

considered to be non-pathogenic and non-toxigenic. Risk group 1 organisms are those not associated with disease in healthy adult humans.

An evaluation of the genetically modified production microorganism for the maltogenic alpha-amylase, embodying the concepts initially outlined by Pariza and Foster, 1983 (2) and further developed by IFBC in 1990 (4), the EU SCF in 1991 (5), the OECD in 1992 (1), ILSI Europe Novel Food Task Force in 1996 (8), FAO/WHO in 1996 (7), JECFA in 1998 (13), Pariza and Johnson in 2001 (3) and Sewalt, et.al. (23) demonstrates the safety of this genetically modified production microorganism strain. The components of this evaluation: the identity of the host 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 Parts 2 and 3.

The genetic modifications are well characterized and specific utilizing well-known plasmids for the vector constructs, and the introduced genetic material does not encode and express any known harmful or toxic substances. Based on the information presented above it is concluded that the *Bacillus subtilis* production strain is considered a safe strain for the production of the maltogenic alpha-amylase enzyme.

The *Bacillus subtilis* strain, A164Δ5, used in the construction of this maltogenic alpha-amylase production strain has previously been used as a host strain in several production strains for Novozymes enzyme products. These production strains were constructed by standard transformation procedures using well-known plasmid vectors and well characterized DNA sequences that were integrated into the *Bacillus subtilis* strain chromosome by homologous recombination.

The safety of this *Bacillus subtilis* production strain was established following published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients (3) (4). The host strain, *Bacillus subtilis,* has been thoroughly characterized as shown in Part 2.2. The introduced DNA is well-known and characterized in Parts 2.3 and 2.4 and the introduced genetic material does not encode and express any known harmful or toxic substances.

Novozymes' used the decision tree (Appendix 1) in Pariza and Johnson 2001 (3) as a basis for our safety assessment. The production strain is genetically modified by rDNA techniques as discussed in Part 2. The expressed maltogenic alpha-amylase enzyme preparation is free of DNA encoding transferable antibiotic resistance gene DNA. The introduced DNA is well characterized and safe for the construction of microorganisms to be used in the production of food grade products. The DNA is stably integrated into the chromosome and the incorporated DNA is known not to encode or express any harmful or toxic substances. The procedures used to modify the host organism are well defined and commonly used. Therefore, the elements needed to establish a safe strain lineage as defined in Pariza and Johnson, 2001 (3) have been met.

Finally, the production strain is derived from a safe lineage. Novozymes has used *Bacillus subtilis* production strains for over 20 years. Safety studies including 13-week oral toxicity in rats, *in vitro* test for gene mutations in bacteria (Ames) and *in vitro* test for chromosomal aberration (in vitro micronucleus assay) have been performed on enzyme preparations from these production strains. No toxicological effects were ever observed for any of the test substances produced by these *Bacillus subtilis* production strains.

6(b) 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 maltogenic alpha-amylase is *Bacillus stearothermophilus*. As indicated in Part 2 the introduced DNA is well defined and characterized. Only well characterized DNA fragments, limited solely to the maltogenic alpha-amylase coding sequence from the donor strain, are used in the construction of the genetically modified strain. The introduced DNA does not code for any known harmful or toxic substances.

6(c) Safety of the Maltogenic Amylase Enzyme

As indicated in Part 2, the subject of this GRAS notification is a maltogenic amylase. Alpha-amylases are widespread in nature and are universally distributed throughout the animal, plant and microbial kingdom (bacteria and fungi). Amylases are naturally present in many raw materials including wheat, barley and malt (24).

Also, the safety of the maltogenic alpha-amylase was assessed in a battery of toxicology studies investigating sub-chronic toxicity and mutagenic potential. The studies show no adverse effects. See Appendix 2 for more detailed information.

A literature search was performed in November 2017 utilizing Medline, ToxCenter, Chemlist, Embase and SciSearch search engines, using search terms “maltogenic alpha- amylase” in combination with the terms “toxicology” and “safety” and “human consumption” and “exposure”, resulting in 92 hits. Novozymes reviewed the available abstracts and found none to be inconsistent with our conclusion of the general recognition of safety for the maltogenic alpha-amylase enzyme.

A wide variety of enzymes are used in food processing and enzyme proteins do not generally raise safety concerns (3) (2). Pariza and Foster note that very few toxic agents have enzymatic properties.

Based on these toxicological data and the fact that the production strain reportedly has a history of safe use, it is our conclusion that the maltogenic alpha-amylase is safe for its intended use as a processing aid in various food applications.

6(d) Allergenic/Toxigenic Potential of the Maltogenic Alpha-Amylase 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 part per millions. The enzyme is typically removed or denatured during food processing, and denatured protein has been shown to be very susceptible to digestion in the gastro-intestinal system. Moreover, a wide range of naturally occurring food enzymes have been shown to be very labile in the gastro-intestinal system even in the native unprocessed form.

The above statements are further supported by the publication: "Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry" (Bindslev-Jensen *et al*, 2006) (25).

In order to further evaluate the possibility that the maltogenic alpha-amylase 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 (26) and modified by Codex Alimentarius Commission, 2009 (14) the maltogenic alpha-amylase was compared to allergens from the FARRP allergen protein database (http://allergenonline.org) as well as the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee (**http://www.allergen.org**).

A search for more than 35% identity in the amino acid sequence of the expressed protein using a window of 80 amino acids and a gap penalty was done. Also, an alignment of the maltogenic alpha-amylase to each of the allergens and identity of hits with more than 35% identity over the full length of the alignment was analyzed.

The analyses identified one mosquito (Aed a 4) and two fungal (Asp o 21 and Sch c 1) allergens, having an identity with maltogenic alpha-amylase above the threshold of 35% across an 80-amino acid window.

None of the hits, Asp o 21, Sch c 1 and Aed a 4, are registered as food allergens (**http://www.allergen.org**). Further, an additional screen of the current literature did not find any evidence that Sch c 1 or Aed a 4 can trigger oral sensitization. The Asp o

21 alpha-amylase very rarely causes oral sensitization and only few cases of potential food allergy to the ingested Asp o 21 alpha-amylase have been described whereof, three were linked to occupational sensitization. (27) (28) (29) (30).

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. In this paper, Dauvrin and colleagues conclude that enzyme exposure by ingestion, in opposition to exposure by inhalation, is extremely unlikely to lead to sensitization. There is compelling evidence that persons affected by occupational asthma can ingest the respiratory allergen without acquiring clinical symptoms of food allergy, suggesting that inhalation is not likely to result in food allergy. Only one single case has been reported in the literature and this case was not verified as a bona fide oral sensitization to enzymes in food (31).

This is backed up by the study conducted by Bindslev-Jensen et al (25) using the generally recognized guidelines for food allergy diagnosis (skin prick test, specific serum IgE and DBPCFC). This study included 400 patients with a 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 (including alpha-amylases) could be found. Further, there were no indications of cross-reactivity between the tested enzymes used in food and the main known allergens causing clinical symptoms in the patients included in the study.

Also, a search for 100% identity over 8 contiguous amino acids was completed. No homology was found. And, a search for homology of the maltogenic alpha-amylase sequence to known toxins was assessed based on the information present in the UNIPROT database (05-03-17). This database contains entries from SWISSPROT and TREMBL. The homology among the emerging entries was below 17%, indicating that the homology to any toxin sequence in this database is low and random.

On the basis of the available evidence and supporting scientific literature, it is concluded that oral intake of maltogenic alpha-amylase produced by *Bacillus subtilis* strain is not anticipated to pose any food allergenic concern.

6(e) Safety of the Manufacturing Process

This section describes the manufacturing process for the maltogenic alpha-amylase, which follows standard industry practices (9) (10) (11). The quality management system used in the manufacturing process for the maltogenic alpha-amylase complies with the requirements of ISO 9001. It is manufactured in accordance with current Good Manufacturing Practices, using ingredients that are accepted for general use in foods, and under conditions that ensure a controlled fermentation. The enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in the Food Chemicals Codex (12). It also conforms to the

General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (13).

6(f) Safety Studies

This section describes the studies and analysis performed to evaluate the safety of the maltogenic alpha-amylase enzyme preparation.

The following studies were performed on enzyme concentrate test batch PPY7087 with favourable results:

- Reverse Mutation Assay (Ames test)
- *in vitro* Chromosome aberration assay
- 14-day oral toxicity study – dose range finder
- 13-week sub-chronic oral toxicity study

These tests are described in Appendix 2. Based on the presented toxicity data and the history of safe use for the strain it can be concluded that maltogenic alpha-amylase represented by batch PPY7087, exhibits no toxicological effects under the experimental conditions described.

6(g) Results and Conclusion

Results of the toxicity and mutagenicity tests described in Appendix 2 showed no toxicity or mutagenicity of the maltogenic alpha-amylase from *Bacillus subtilis*, PPY7087. A critical review and evaluation of the maltogenic alpha-amylase from *Bacillus subtilis* was done following the concepts of the Pariza papers (2) (3) and the recently described process for the evaluation of GRAS for industrial microbial enzymes by Sewalt et al. (23).

Based on known history of safe use of the production organism and the limited and well defined nature of the genetic modifications, Novozymes concludes through scientific procedures that the subject of this notification; maltogenic alpha-amylase enzyme preparation is generally recognized, among qualified experts, to be safe under the conditions of its intended use**.**

PART 7 – SUPPORTING DATA AND INFORMATION

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

APPENDICES

1. Pariza and Johnson Decision Tree Analysis

2. Summary of Toxicity Data, Maltogenic alpha-amylase, batch PPY7087. February 2002, LUNA No. 2002-03563-01.

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

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

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

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

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

(b) (6)

12/18/17

Janet Oesterling
Regulatory Affairs Specialist III

Date

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(b) (6)

12/18/17

Janet Oesterling
Regulatory Affairs Specialist III

Date

Appendix 3- This maltogenic alpha-amylase from *Bacillus stearothermophilus* produced by a genetically modified strain of *Bacillus subtilis* was evaluated according to the decision tree published in Pariza and Johnson, 2001 [(1)].
The result of the evaluation is presented below.

Decision Tree

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

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

3. Issues relating to the introduced DNA are addressed in 3a-3e.
   a. Does the expressed enzyme product which is encoded by the introduced DNA have a history of safe use in food?
      YES, go to 3c**.**

   c. Is the test article free of transferable antibiotic resistance gene DNA?
      YES, go to 3e**.**

   e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food products?
      YES*,* *go to 4.*

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

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?
   YES. ***If yes the test article is ACCEPTED****.*

LIST OF REFERENCES

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

Toxicology
Date: February 2002
Ref: DSBR
File: 2002-03563-01

SUMMARY OF TOXICITY DATA

Maltogenic alpha-amylase batch PPY7087 produced by *Bacillus subtilis*

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CONTENTS

1. ABSTRACT

Test batch, PPY 7087, is a liquid enzyme concentrate. It is produced by submerged fermentation of a strain of *Bacillus subtilis*, containing the gene code of a bacterial amylolytic enzyme originating from *Bacillus stearothermophilus.*

In the following, toxicity studies carried out are summarised.

All studies were carried out in accordance with current EU and OECD guidelines and in compliance with the OECD principles of Good Laboratory Practice (GLP). The studies were carried out at Scantox, Denmark or Covance Laboratories, England during the period March 2001 to January 2002.

The main conclusions of the safety studies can be summarised as follows:

Oral administration of PPY 7087 to SPF Sprague Dawley rats at dosages of up to 10.0 mL/kg bw/day (equivalent to 968.2 mg TOS/kg bw/day or 91504 MANU/kg bw day) for 14-days was well tolerated and produced no toxicological significant findings.

Oral administration of PPY 7087 to rats at dosages up to 10 mL/kg body weight/day for 13 weeks was well-tolerated and did not produce any toxicological significant changes. Consequently, 10 mL/kg/day day (equivalent to 91504 MANU/kg/day or 968.2 mg TOS/kg/day) was considered to be the No-Observed-Adverse-Effect Level (NOAEL) in this study.

PPY 7087 has shown no mutagenic activity in either Ames' test or the chromosome aberration test.

2. TEST SUBSTANCE

2.1 Production of test substance

PPY 7087, is a liquid enzyme concentrate. The principal enzyme activity is a maltogenic amylase classified as Glucan 1,4-a-maltohydrolase (EC 3.2.1.133). It acts on starch and related polysaccharides and oligosaccharides by hydrolysis of (1->4)-alpha-D-glucosidic linkages. The enzyme originates from a strain of *Bacillus stearothermophilus* and is expressed in a strain of *Bacillus subtilis.* The product is produced by submerged fermentation and recovered by purification/ concentration of the fermented culture broth.
The batch PPY 7087, used for the present toxicological programme, was a mixture of 3 identically produced sub-batches.

2.2 Characterisation

Batch PPY 7087, a dark brown liquid was used for all the studies.

Table 1. Characterisation of PPY 7087

	Batch No. PPY 7087
Activity, MANU/g	8600
Water (KF)	86.1%
Dry matter	13.9%
Ash (600°C)	4.8%
Total Organic Solids (TOS[1])	9.1%
Specific gravity (g/mL)	1.064

[1] %TOS = 100%- % water- % ash- % diluents

2.3 Production Microorganism

The production organism is produced by a strain of *Bacillus subtilis*, containing the maltogenic amylase gene code originating from *Bacillus stearosthermophilus.* This genetically modified production strain meets the criteria for a safe production microorganism. It is constructed by common transformation procedures using well-known plasmid vectors with strictly defined and well-characterized DNA sequences that are not known to encode or express any harmful or toxic substances. The strain is free of any antibiotic resistance marker. The development of the production strain was evaluated at every step to assess incorporation of the desired functional genetic information and to ensure no unintended sequences were incorporated.

Bacillus subtilis has long history of safe use. This species has been used for decades in the production of enzymes, and in the last decade as recombinant organism for production of a variety of bio-industrial products.

Bacillus subtilis is generally regarded as non-pathogenic and non-toxigenic. Further investigations have revealed that the current production strain line does not produce any known *Bacillus* toxins.

The test substance does not contain the production strain. Absence of the production strain is part of the complete specification of the product.

3. TOXICITY DATA

3.1 Subacute Toxicity

3.1.1 A 14-Day Oral (Gavage) Dose-Range-Finder Study in Rats

A sub-acute toxicity study in rats with a duration of two weeks served as a dose range finding study to the 13-week study.

Three groups each of five male and five female SPF Sprague Dawley rats received PPY 7087 orally by gavage, at dosages of 1.0 mL, 3.3 mL or 10.0 mL/kg bw/day (equivalent to 96.8, 319.5 or 968.2 mg TOS/kg bw/day, or 9150.4, 30196.3 or 91504 MANU/kg bw/day). A similar constituted group received the vehicle (tap water) and served as the negative control. The dose volume was 10 mL/kg bw/day.

On day 1 and 8 of treatment, samples from each dose formulation were taken and analysed for achieved concentration. Achieved concentration was evaluated by measurement of enzyme activity, MANU/g.

At the end of 2 weeks treatment, all animals were killed and subjected to necropsy.

No clinical signs were observed. Body weight and food consumption were unremarkable. No abnormal observations at necropsy.
The results from the content check analysis showed that the measured activity was slightly higher than expected, so it can be concluded that the animals were dosed with at least the expected amount of test substance.

It was concluded that daily oral (gavage) administration of PPY 7087 to SPF Sprague Dawley rats at dosages of up to 10.0 mL/kg bw/day (equivalent to 968.2 mg TOS/kg bw/day or 91504 MANU/kg bw day) for 14-days was well tolerated and produced no toxicological significant findings. Consequently, the NOAEL (No Observed Adverse Effect Level) was at or above 968.2 mg TOS/kg bw PPY 7087, based on data after daily dosing for 14 days

3.2. **Subchronic Toxicity**

3.2.1. A 13-week Oral (Gavage) Toxicity Study in Rats

The study was carried out in accordance with the OECD guideline 408 (adopted on September 1998). It was conducted in accordance with Good Laboratory Practice.
Three groups of 10 male and 10 female rats were dosed, PPY 7087 by gavage at dosages of 1.0, 3.3 or 10.0 mL/kg bw/day. This corresponds to 9150, 30196 or 91504 MANU/kg/day or 96.8, 319.5 or 968.2 mg TOS/kg/day. A similar constituted group received the vehicle (tap water) and served as the negative control.

Analysis of achieved concentration was performed on samples taken once during weeks 1, 6 and 13. Achieved concentration was evaluated by measurement of enzyme activity, MANU/g.

The clinical observations revealed no treatment-related effects. No differences were seen between treated and control animals in clinical signs, body weight gain, food consumption, opthalmoscopy, haematology, clinical chemistry, and urinalysis or organ weight. There were no macroscopic or microscopic findings that could be related to treatment with the test substance.

The results from the content check analysis showed that in the samples from week 1 and 13 there was no proofed difference between the intended activity and the results. The results from week 6 were only slightly lower than expected considering the coefficient of variation for the method (5.2 %).

It was concluded that the oral administration of PPY 7087 to rats at dosages up to 10 mL/kg body weight/day for 13 weeks was well-tolerated and did not produce any toxicological significant changes. Consequently, 10 mL/kg/day day (equivalent to 91504 MANU/kg/day or 968.2 mg TOS/kg/day) was considered to be the No-Observed-Adverse-Effect Level (NOAEL) in this study.

3.3. **Mutagenic Potential**

3.3.1. Bacterial Reverse Mutation Assay

PPY 7087 was examined in a bacterial reverse mutation assay in accordance with OECD Guideline for Testing of Chemicals No. 471 (1997) in order to determine the ability to induce gene mutations in bacteria.

Four histidine-requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and one tryptophan-requiring strain of *Escherichia coli* (WP2 uvrA) were applied in this study.

The study was conducted, using the direct plate incorporation assay, in the presence and absence of metabolic activation - a liver preparation from male rats, pre-treated with Aroclor1254, and the co-factors required for mixed function oxidase activity (S9 mix).

Each test with each strain was prepared on two separate occasions. In both these experiments bacteria were exposed to 6 doses separated with bi-sections. The highest dose level applied was 5 mg.

The sensitivity of the individual bacterial strains was confirmed by incorporation of diagnostic mutagens. In all test series these positive control substances induced significant and satisfactory responses in the appropriate strains in similar conditions as the test article.

No significant, dose-related and reproducible increases in reversion to prototrophy were obtained with any of the bacterial strains exposed to batch PPY 7087, either in the presence or absence of S9 mix.

It was concluded, that the results of the experiments, described in this report, give no indication of mutagenic activity of PPY 7087, in the presence or absence of metabolic activation, when tested under the conditions employed in this study.

3.3.2 **Chromosome Aberration Assay**

The effects on chromosomal structure of exposure to PPY 7087 were investigated in cultured human lymphocytes in accordance with the current guidelines of OECD (Guideline 473, July 1997).

Heparinized whole blood cultures, pooled from three female donors, were established, and division of the lymphocytes was stimulated by adding phytohaemagglutinin (PHA) to the cultures.
Two independent experiments were performed both in the absence and presence of metabolic activation by a rat liver post-mitochondrial fraction (S-9) from animals induced with Aroclor. Sets of duplicate cultures were treated with the solvent (sterile purified water), test chemical or positive controls (–S-9: 4-Nitroquinoline 1-oxide, +S-9: Cyclophosphamide).

Treatments with PPY 7087 covered a broad range of doses, separated by narrow intervals, where the highest dose level used was 5000 μg/mL.
The lymphocyte cultures were exposed to the test substance for 3 hours and cells were harvested 17 hours later. The second experiment included a continuous exposure for 20 hours in the absence of S-9. The test article dose levels for chromosome analysis were selected by evaluating the effect of PPY 7087 on mitotic index.
Chromosome aberrations were analysed at three consecutive dose levels. Cells were arrested in metaphase by colchicine and after centrifugation and hypotonic treatment metaphase spreads were prepared and stained with Giemsa. A total of 200 cells were scored per dose level (100 from each replicate culture) from PPY 7087 treatments and negative controls. Slides were scored blind and aberrations recorded according to international classifications.
The proportion of cells with structural aberrations in all cultures of the solvent controls (purified water) was within the limits of the historical ranges. The positive controls induced statistically significant increases in the proportion of cells with structural aberrations, thus demonstrating the sensitivity of the test procedure and the metabolic activity of the S-9 mix employed.
In the first experiment the highest concentration of PPY 7087 chosen for analysis, 5000 μg/mL, produced no mitotic inhibition (reduction in mitotic index) in the absence and presence of S-9, respectively. In the second experiment the highest concentration chosen for analysis, 3071 and 5000 μg/mL produced approximately 47% and 1% mitotic inhibition in the absence and presence of S-9, respectively.

Cells treated with PPY 7087 either in the absence and presence of S-9, had similar numbers of aberrations to those observed in concurrent solvent controls. There were no reproducible increases in aberration frequency that were significantly higher than those observed in the negative controls or that fell outside the historical negative control ranges.
Normal frequencies of cells with numerical aberrations were seen under all treatment conditions.

Under the conditions of test PPY 7087 did not induce chromosome aberrations in cultured human blood lymphocytes when tested to a concentration of 5000 μg/mL in either the absence or presence of S-9.

4. CONCLUSION

The results of the toxicological studies outlined above did not reveal any adverse effect of batch PPY 7087. Based on these toxicological data and the fact that the production strain reportedly has a history of safe use, it is our conclusion that the maltogenic amylase is safe for its intended use as a processing aid in various food applications.

5. REFERENCES

5.1 Study Reports

Scantox: Lab No.: 42460. Novozymes Reference No.: 20016015.
PPY 7087: A 14-Day Oral (Gavage) Dose-Range-Finder Study in Rats. September 2001.

Scantox: Lab No No.: 42461. Novozymes Reference No.: 20016016.
PPY 7087: A 13-week Oral (Gavage) Toxicity Study in Rats. January 2002.

Novozymes A/S Report No.: 2001-13295-01. NN Study No.: 20018041. (Batch No. PPY 7087): Test for mutagenic activity with strains of *Salmonella typhimurium* and *Escherichia coli.*

Covance Laboratories Limited Report No.1974/8. Novozymes Study No.20016020
Lipase: Induction of Chromosome Aberrations in Cultured Human Peripheral Blood Lymphocyte.

5.2 Guidelines

EEC, 1992. Annex to Commission Directive 92/69/EEC. *The Journal of the European Communities* L383A, 29 December.

OECD, Guidelines for testing of Chemicals. Section 3 and 4: Health effects. Organisation for Economic Co-operation and Development, Paris.

OECD principles of Good Laboratory Practice (GLP) (as revised in 1997), ENV/MC/CHEM(98)17. OECD, Paris.

Toxicology
Date: February 2002
Ref: DSBR
File: 2002-03563-01

SUMMARY OF TOXICITY DATA

Maltogenic alpha-amylase batch PPY7087 produced by *Bacillus subtilis*

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CONTENTS

1. ABSTRACT

Test batch, PPY 7087, is a liquid enzyme concentrate. It is produced by submerged fermentation of a strain of *Bacillus subtilis*, containing the gene code of a bacterial amylolytic enzyme originating from *Bacillus stearothermophilus.*

In the following, toxicity studies carried out are summarised.

All studies were carried out in accordance with current EU and OECD guidelines and in compliance with the OECD principles of Good Laboratory Practice (GLP). The studies were carried out at Scantox, Denmark or Covance Laboratories, England during the period March 2001 to January 2002.

The main conclusions of the safety studies can be summarised as follows:

Oral administration of PPY 7087 to SPF Sprague Dawley rats at dosages of up to 10.0 mL/kg bw/day (equivalent to 968.2 mg TOS/kg bw/day or 91504 MANU/kg bw day) for 14-days was well tolerated and produced no toxicological significant findings.

Oral administration of PPY 7087 to rats at dosages up to 10 mL/kg body weight/day for 13 weeks was well-tolerated and did not produce any toxicological significant changes. Consequently, 10 mL/kg/day day (equivalent to 91504 MANU/kg/day or 968.2 mg TOS/kg/day) was considered to be the No-Observed-Adverse-Effect Level (NOAEL) in this study.

PPY 7087 has shown no mutagenic activity in either Ames' test or the chromosome aberration test.

2. TEST SUBSTANCE

2.1 Production of test substance

PPY 7087, is a liquid enzyme concentrate. The principal enzyme activity is a maltogenic amylase classified as Glucan 1,4-a-maltohydrolase (EC 3.2.1.133). It acts on starch and related polysaccharides and oligosaccharides by hydrolysis of (1->4)-alpha-D-glucosidic linkages. The enzyme originates from a strain of *Bacillus stearothermophilus* and is expressed in a strain of *Bacillus subtilis.* The product is produced by submerged fermentation and recovered by purification/ concentration of the fermented culture broth.
The batch PPY 7087, used for the present toxicological programme, was a mixture of 3 identically produced sub-batches.

2.2 Characterisation

Batch PPY 7087, a dark brown liquid was used for all the studies.

Table 1. Characterisation of PPY 7087

	Batch No. PPY 7087
Activity, MANU/g	8600
Water (KF)	86.1%
Dry matter	13.9%
Ash (600°C)	4.8%
Total Organic Solids (TOS[1])	9.1%
Specific gravity (g/mL)	1.064

[1] %TOS = 100%- % water- % ash- % diluents

2.3 Production Microorganism

The production organism is produced by a strain of *Bacillus subtilis*, containing the maltogenic amylase gene code originating from *Bacillus stearosthermophilus.* This genetically modified production strain meets the criteria for a safe production microorganism. It is constructed by common transformation procedures using well-known plasmid vectors with strictly defined and well-characterized DNA sequences that are not known to encode or express any harmful or toxic substances. The strain is free of any antibiotic resistance marker. The development of the production strain was evaluated at every step to assess incorporation of the desired functional genetic information and to ensure no unintended sequences were incorporated.

Bacillus subtilis has long history of safe use. This species has been used for decades in the production of enzymes, and in the last decade as recombinant organism for production of a variety of bio-industrial products.

Bacillus subtilis is generally regarded as non-pathogenic and non-toxigenic. Further investigations have revealed that the current production strain line does not produce any known *Bacillus* toxins.

The test substance does not contain the production strain. Absence of the production strain is part of the complete specification of the product.

3. TOXICITY DATA

3.1 Subacute Toxicity

3.1.1 A 14-Day Oral (Gavage) Dose-Range-Finder Study in Rats

A sub-acute toxicity study in rats with a duration of two weeks served as a dose range finding study to the 13-week study.

Three groups each of five male and five female SPF Sprague Dawley rats received PPY 7087 orally by gavage, at dosages of 1.0 mL, 3.3 mL or 10.0 mL/kg bw/day (equivalent to 96.8, 319.5 or 968.2 mg TOS/kg bw/day, or 9150.4, 30196.3 or 91504 MANU/kg bw/day). A similar constituted group received the vehicle (tap water) and served as the negative control. The dose volume was 10 mL/kg bw/day.

On day 1 and 8 of treatment, samples from each dose formulation were taken and analysed for achieved concentration. Achieved concentration was evaluated by measurement of enzyme activity, MANU/g.

At the end of 2 weeks treatment, all animals were killed and subjected to necropsy.

No clinical signs were observed. Body weight and food consumption were unremarkable. No abnormal observations at necropsy.
The results from the content check analysis showed that the measured activity was slightly higher than expected, so it can be concluded that the animals were dosed with at least the expected amount of test substance.

It was concluded that daily oral (gavage) administration of PPY 7087 to SPF Sprague Dawley rats at dosages of up to 10.0 mL/kg bw/day (equivalent to 968.2 mg TOS/kg bw/day or 91504 MANU/kg bw day) for 14-days was well tolerated and produced no toxicological significant findings. Consequently, the NOAEL (No Observed Adverse Effect Level) was at or above 968.2 mg TOS/kg bw PPY 7087, based on data after daily dosing for 14 days

3.2. **Subchronic Toxicity**

3.2.1. A 13-week Oral (Gavage) Toxicity Study in Rats

The study was carried out in accordance with the OECD guideline 408 (adopted on September 1998). It was conducted in accordance with Good Laboratory Practice.
Three groups of 10 male and 10 female rats were dosed, PPY 7087 by gavage at dosages of 1.0, 3.3 or 10.0 mL/kg bw/day. This corresponds to 9150, 30196 or 91504 MANU/kg/day or 96.8, 319.5 or 968.2 mg TOS/kg/day. A similar constituted group received the vehicle (tap water) and served as the negative control.

Analysis of achieved concentration was performed on samples taken once during weeks 1, 6 and 13. Achieved concentration was evaluated by measurement of enzyme activity, MANU/g.

The clinical observations revealed no treatment-related effects. No differences were seen between treated and control animals in clinical signs, body weight gain, food consumption, opthalmoscopy, haematology, clinical chemistry, and urinalysis or organ weight. There were no macroscopic or microscopic findings that could be related to treatment with the test substance.

The results from the content check analysis showed that in the samples from week 1 and 13 there was no proofed difference between the intended activity and the results. The results from week 6 were only slightly lower than expected considering the coefficient of variation for the method (5.2 %).

It was concluded that the oral administration of PPY 7087 to rats at dosages up to 10 mL/kg body weight/day for 13 weeks was well-tolerated and did not produce any toxicological significant changes. Consequently, 10 mL/kg/day day (equivalent to 91504 MANU/kg/day or 968.2 mg TOS/kg/day) was considered to be the No-Observed-Adverse-Effect Level (NOAEL) in this study.

3.3. **Mutagenic Potential**

3.3.1. Bacterial Reverse Mutation Assay

PPY 7087 was examined in a bacterial reverse mutation assay in accordance with OECD Guideline for Testing of Chemicals No. 471 (1997) in order to determine the ability to induce gene mutations in bacteria.

Four histidine-requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and one tryptophan-requiring strain of *Escherichia coli* (WP2 uvrA) were applied in this study.

The study was conducted, using the direct plate incorporation assay, in the presence and absence of metabolic activation - a liver preparation from male rats, pre-treated with Aroclor1254, and the co-factors required for mixed function oxidase activity (S9 mix).

Each test with each strain was prepared on two separate occasions. In both these experiments bacteria were exposed to 6 doses separated with bi-sections. The highest dose level applied was 5 mg.

The sensitivity of the individual bacterial strains was confirmed by incorporation of diagnostic mutagens. In all test series these positive control substances induced significant and satisfactory responses in the appropriate strains in similar conditions as the test article.

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It was concluded, that the results of the experiments, described in this report, give no indication of mutagenic activity of PPY 7087, in the presence or absence of metabolic activation, when tested under the conditions employed in this study.

3.3.2 **Chromosome Aberration Assay**

The effects on chromosomal structure of exposure to PPY 7087 were investigated in cultured human lymphocytes in accordance with the current guidelines of OECD (Guideline 473, July 1997).

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The lymphocyte cultures were exposed to the test substance for 3 hours and cells were harvested 17 hours later. The second experiment included a continuous exposure for 20 hours in the absence of S-9. The test article dose levels for chromosome analysis were selected by evaluating the effect of PPY 7087 on mitotic index.
Chromosome aberrations were analysed at three consecutive dose levels. Cells were arrested in metaphase by colchicine and after centrifugation and hypotonic treatment metaphase spreads were prepared and stained with Giemsa. A total of 200 cells were scored per dose level (100 from each replicate culture) from PPY 7087 treatments and negative controls. Slides were scored blind and aberrations recorded according to international classifications.
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From: JAO (Janet Oesterling) <JAO@novozymes.com>
Sent: Wednesday, July 18, 2018 11:22 AM
To: Highbarger, Lane A
Subject: Maltogenic amylase - GRN 751

Hello Lane,

Thank you for calling today regarding questions on maltogenic amylase, GRN 751. Below is our response to those questions. Please let me know if you need anything else.

QUESTION: In section 2.2(d), the chloramphenicol resistance marker was removed. When was it put in?

ANSWER: The chloramphenicol resistance gene was introduced at the same time as the amyM expression cassette and was used as a selective marker to select the transformants having integrated in their genome the amyM expression cassette. Therefore, only the chloramphenicol resistance gene was then removed in the consecutive GM step after integration of the amyM expression cassette leading to a strain expressing the amyM gene and without the chloramphenicol resistance marker.

QUESTION: In Section 2.2(c), you mention “The plasmid contains the expression cassette consisting of the Bacillus amyloliquefaciens and Bacillus thuringiensis engineered promoter, the amyM coding sequence and a transcriptional terminator.” What does this mean?

ANSWER: We mean, by using the term “expression cassette” the following genetic elements : a promoter, a gene and a terminator. In the case of the amyM expression cassette it consists of an engineered promoter derived from both B. amyloliquefaciens and B. thuringiensis, the amyM gene from B. stearothermophilus coding for the maltogenic amylase and a transcriptional terminator.

Best regards,

Janet Oesterling
Regulatory Affairs Specialist III

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From:	JAO (Janet Oesterling)
To:	Highbarger, Lane A
Subject:	Maltogenic amylase - GRN 751
Date:	Wednesday, July 18, 2018 11:36:43 AM
Attachments:	image001.gif

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Best regards,

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