ORIGINAL SUBMISSION

June 3, 2016

GRAS Notification Program
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
US Food And Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Sir or Madam,

We are hereby submitting via the ESG Gateway, a generally recognized as safe (GRAS) notification, in accordance with proposed 21 C.F.R. § 170.36 (a), for Novozymes' phospholipase A1 enzyme preparation produced by a genetically modified strain of *Aspergillus niger.*

Novozymes has determined through scientific procedures that the glucoamylase is generally recognized as safe for use in the food industry as a processing aid in starch processing.

Please contact me by direct telephone at 919 494-3187, direct fax at 919 494-3420 or email at jao@novozymes.com if you have any questions or require additional information.

Sincerely,

(b) (6)

Janet Oesterling
Regulatory Affairs Specialist III

Enclosures

Novozymes North America, Inc.
Regulatory Affairs
77 Perry Chapel Church Road, P.O. Box 576
Franklinton, North Carolina 27525

Tel:919-494-3000 ***Fax: 919-494-3420*** ***www.novozymes.com***

PART III – GENERAL ADMINISTRATIVE INFORMATION

1. Name of Substance

Phospholipase A preparation produced by a genetically modified strain of Aspergillus niger

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 intended use in starch processing. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following cGMP. The maximum recommended use level is 3200 AGU per kg of starch based raw material in solid food and 3000 AGU of starch based raw material in liquid food. No special population.

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

000004

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	Glucoamylase	CAS	EC No: 3.2.1.3		
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.

Glucoamylases Hydrolyze 1,4-alpha as well as 1,6-alpha linkages in liquefied starch. During hydrolysis, the glucoamylase activity removes glucose units in a stepwise manner from the non-reducing end of the substrate molecule.

3. Synonyms

Provide as available or relevant:

1	Glucan 1,4-alpha glucosidase
2	
3	

000005

PART VI – OTHER ELEMENTS IN YOUR GRAS NOTICE

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

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

Other Information

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

☒ Yes ☐ No

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

☒ Yes ☐ No

PART VII – SIGNATURE

1. The undersigned is informing FDA that janet oesteling
(name of notifier)

has concluded that the intended use*(s)* of Phospholipase A preparation produced by a genetically modified strain of Aspergillus niger
(name of notified substance)

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

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

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

(address of notifier or other location)

janet oesteling *(name of notifier)* agrees to send these data and information to FDA if FDA asks to do so.

OR

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

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
janet oesterling Digitally signed by janet oesterling Date: 2016.06.03 14:26:05 -04'00'	Janet Oesterling, Regulatory Specialist III	06/03/2016

000006

PART VIII – LIST OF ATTACHMENTS

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

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Form3667_PhospholipaseA1_2016-05-05.pdf	Incoming Correspondence/Submission Form
	CoverLetter_PhospholipaseA1_2016-05-05.pdf	Administrative
	Claimletter_PhospholipaseA1_2016-05-05.pdf	Administrative
	GRASNotification_PhospholipaseAfromAspergillusniger_2016-05-05.pdf	Submission
	Safetyof MicrobialEnzymePreps_ParizaandJohnson_April2001.pdf	Administrative
	SummaryofToxicityData_PhospholipaseA1_2016-05-03.pdf	Administrative
	DecisionTree_PhospholipaseA1_2016-05-05.pdf	Administrative

June 3, 2016

RE: GRAS Notification - Exemption Claim

Dear Sir or Madam:

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

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

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

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

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

Glucoamylase enzyme preparation produced by a genetically modified *Aspergillus niger.*

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

The glucoamylase is used as a processing aid in starch processing. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following Good Manufacturing Practices.

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

This GRAS determination is based on scientific procedures.

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

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

(b) (6)

05-02-16

Janet Oesterling Date
Regulatory Affairs Specialist III

A Glucoamylase preparation produced by a genetically modified strain of *Aspergillus niger*

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

June 2016

1. GENERAL INTRODUCTION

The subject of this notification is a glucoamylase enzyme preparation produced by submerged fermentation of a genetically modified *Aspergillus niger* microorganism carrying the gene coding for glucoamylase from *Penicillum oxalicum.*

This glucoamylase enzyme preparation is intended for use as a processing aid in food raw materials which contain starch. The enzyme converts starch and hydrolysis products of starch into D-glucose and can be of benefit in the processing of all foods and food ingredients which naturally contain the substrate.

The active enzyme is glucoamylase (EC 3.2.1.3, CAS 9032-08-0).

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

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food (1) (2) (Appendix 1). The production organism for the glucoamylase, *Aspergillus niger,* is discussed in Sections 2 and 7. *Aspergillus niger* has a documented history of safe use in food and is considered to be a safe organism for the production of enzymes or ingredients used in food (3).

Aspergillus niger was exempted from EPA review under TSCA (4). *Aspergillus niger* has a long history of safe use. The FDA has previously affirmed as GRAS several enzyme preparations from *Aspergillus niger* (GRAS notifications 000089, 000111, 000132) to name a few (5).

Aspergillus niger is generally considered to be non-pathogenic and non-toxigenic and is often mentioned as an example of a well characterized and safe production strain with a long history of safe use. *Aspergillus niger* meets the US Environmental Protection Agency (EPA) criteria for non-toxigenicity and non-pathogenicity,and is one of 10 recipient organisms eligible for Tier I exemption under the EPA regulations (6).

The production organism is derived from the *Aspergillus niger* recipient strain. *Aspergillus niger* has been used by industry since 1919 for the production of citric acid which can be an ingredient in foods such as soft drinks, fruit juices and jams. The US Food and Drug Administration (FDA) listed *Aspergillus niger* as a source of citric acid (21 CFR 173.280). This species is well known to produce various enzymes for food use. The Association of Microbial Food Enzyme Producers (AMFEP) listed

Aspergillus niger as safe source of microbial food enzymes (7). In Section 7.1.1, we show the basis for a safe strain lineage for this production strain following the procedure outlined in Pariza and Johnson 2001 (Appendix 1).

An essential aspect of the safety evaluation of food components derived from genetically modified organisms is the identification and characterization of the inserted genetic material (8) (9) (10) (11) (12) (13). The methods used to develop the genetically modified production organism and the specific genetic modifications introduced into the production organism are described in Section 2.

This notification includes information that addresses the safety of the enzyme source, the enzyme component, the manufacturing process and a consideration of dietary exposure which covers all the issues relevant to a safety evaluation of an enzyme preparation. Based on critical review and evaluation of its published and unpublished information, Novozymes concludes through scientific procedures that the subject of this notification, glucoamylase enzyme preparation, meets the appropriate food grade specifications and is produced in accordance with current good manufacturing practices, thus making it GRAS for the intended conditions of use.

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The *Aspergillus niger* production strain, designated 126-PE001 was derived from recipient strain M1371, a natural isolate of *Aspergillus niger* strain BO-1. 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 (10). It also meets the criteria for a safe production microorganism as described by Pariza and Foster (2) and later Pariza and Johnson (1) and several expert groups (8) (9) (10) (11) (12) (13).

The expression plasmid, used in the strain construction, *pIhar220*, contains strictly defined chromosomal DNA fragments and synthetic DNA linker sequences. The DNA sequence for the introduced gene is based on the *amgPO* sequence encoding a glucoamylase (AMG) from *Penicillium oxalicum*.

2.2 Recipient Strain

The recipient strain M1371 used in the construction of the glucoamylase production strain was modified at several chromosomal loci during strain development to inactivate genes encoding a number of amylases and proteases. Furthermore the fumonisin gene cluster and the oxaloacetate hydrolase gene were deleted in M1371 together with the deletion of additional genes encoding unwanted proteins that can be present in the culture supernatant. The lack of these represents improvements in the product purity, safety and stability.

2.3 Glucoamylase (AMG) Expression Plasmid

The expression plasmid, pIhar220, used to introduce a glucoamylase gene in the recipient strain M1371 is based on the replication origin of E. coli. However, no fragments of the vector backbone are introduced into the production strain.

The plasmid contains the expression cassette consisting of a fragment of the *Aspergillus niger* promotor, a sequence encoding a glucoamylase gene, a transcriptional terminator and finally a selective marker; *pyrG* encoding orotidine-5'-phosphate-decarboxylase.

The glucoamylase gene is a genetically engineered variant of the wild-type glucoamylase from *Penicillium* with a single amino acid residue difference compared to the wild type sequence.

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

2.4 Construction of the Recombinant Microorganism

The production strain, *Aspergillus niger* 126-PE001, was constructed from the recipient strain M1371 through the following steps:

The expression cassette from plasmid *pIhar220* was integrated into four specific loci in strain M1371 by targeted homologous recombination to these loci. Targeted integration of the expression cassettes at these loci allows the expression of the glucoamylase gene from the promoter.

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

The resulting glucoamylase (AMG) production strain containing one copy of the glucoamylase gene at each of the four target loci was named 126-PE001.

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

2.5 Stability of the Introduced Genetic Sequences

The genetic stability of the introduced DNA sequences was determined by Southern blot. Analysis of samples from end of production using a *amgPO* gene specific probe showed an identical band pattern compared to the reference production strain (126-

PE001), demonstrating the genetic stability of the introduced DNA during production. The transforming DNA is stably integrated into the *Aspergillus niger* chromosome and, as such, is poorly mobilized for genetic transfer to other organisms and is mitotically stable.

2.6 Antibiotic Resistance Gene

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

2.7 Absence of Production Organism in Product

The absence of the production organism is an established specification for the commercial product. The production organism does not end up in food and therefore the first step in the safety assessment as described by IFBC (8) is satisfactorily addressed.

3. MANUFACTURING PROCESS

This section describes the manufacturing process for the glucoamylase which follows standard industry practices (14) (15) (16). The quality management system used in the manufacturing process for the glucoamylase complies with the requirements of ISO 9001. It is produced under a standard manufacturing process as outlined by Aunstrup (15) and in accordance with current Good Manufacturing Practices, using ingredients that are accepted for general use in foods, and under conditions that ensure a controlled fermentation. The enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in the Food Chemicals Codex (17). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (18).

3.1 Raw Materials

The raw materials used in the fermentation and recovery process for the glucoamylase enzyme concentrate are standard ingredients used in the enzyme industry (14) (15) (16). The raw materials conform to Food Chemicals Codex specifications except those raw materials which do not appear in the FCC. For those not appearing in the FCC, internal specifications have been made in line with FCC requirements. On arrival at Novozymes A/S, the raw materials are sampled by the Quality Control Department and subjected to the appropriate analyses to ensure their conformance to specifications.

Any antifoams or flocculants used in fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams

and flocculants dated April 10, 1998. The maximum use level of the antifoams if used in the product is less than 1%.

3.2 Fermentation Process

The glucoamylase enzyme preparation is produced by pure culture submerged fed-batch fermentation of a genetically modified strain of *Aspergillus niger* as described in Section 2. All equipment is carefully designed, constructed, operated, cleaned, and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are done to ensure absence of foreign microorganisms and confirm strain identity.

3.2.1 Production Organism

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

3.2.2 Criteria for the Rejection of Fermentation Batches

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

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

1) Contamination is observed in 2 or more samples by microscopy

2) Contamination is observed in two successive agar plates at a minimum interval of 6 hours

Any contaminated fermentation is rejected.

3.3 Recovery Process

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

3.3.1 Purification Process

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

1) Pretreatment - pH adjustment and flocculation

2) Primary Separation – vacuum drum filtration or centrifugation

3) Concentration - ultrafiltration and/or evaporation

4) Pre- and Germ Filtration - for removal of residual production strain organisms and as a general precaution against microbial degradation

5) Final concentration – evaporation and/or ultrafiltration.

6) Preservation and Stabilization of the liquid enzyme concentrate

3.3.2 Formulation and Standardization Processes

The powdered enzyme preparation is standardized with sodium chloride and dextrin See Table 1 below.

3.4 Quality Control of Finished Product

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

4. ENZYME IDENTITY

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

Classification	Glucoamylase
IUBMB nomenclature:	Glucan 1,4-alpha glucosidase
EC No.:	3.2.1.3
CAS No.:	9032-08-0
Specificity:	Hydrolyses 1,4-alpha as well as 1,6-alpha linkages in liquefied starch. During hydrolysis, the glucoamylase activity removes glucose units in a stepwise manner from the non-reducing end of the substrate molecule.
Amino acid sequence:	the total nucleotide and amino acid sequences have been determined

6. APPLICATION

6.1 Mode of Action

The active enzyme is a glucoamylase (EC 3.2.1.3). Glucoamylases catalyze the hydrolysis of 1,4-alpha and 1,6-alpha linkages in starch polysaccharides, amylose and amylopectin, and hydrolysis-products of starch such as dextrin. The hydrolysis of 1,4-alpha linkages often proceed at a higher rate compared to hydrolysis of 1,6-alpha linkages.

The glucoamylase preparation is used as a processing aid during food manufacturing. The typical food processes where this glucoamylase is used include baking processes and other cereal based processes, brewing processes and other cereal based beverage processes and starch processing.

In the baking process, glucoamylase is added together with other raw materials during the dough formation step. During processing of baked goods glucoamylase hydrolyses starch and dextrin from starch granules that have been damaged during the milling process into glucose, which can be fermented by yeast. Furthermore, the increased amount of glucose promotes Maillard reactions, which provides more intense browning of the crust during baking.

For other cereal based processes glucoamylase is added to the slurry of milled raw materials and water, which is then heated to facilitate the use of enzymes (e.g. glucoamylases and alpha-amylases) followed by a temperature treatment (pasteurization) step. The final slurry or paste can then undergo different types of processing like extruding or sheeting followed by a baking or frying step for breakfast cereals and snacks, or a homogenization step for cereal containing beverages.

In the brewing process starch and other raw material components are degraded during mashing. The liquid phase (the wort) is recovered from the mash by filtration before the wort is boiled and then fermented with yeast to produce the beer. Starch is the main component of the endosperm of malt, barley, wheat and other cereals. A main purpose of the brewing process (the mashing) is to convert starch into dextrin and fermentable sugars. The glucoamylase can be applied to consistently achieve predictable and targeted attenuation levels which otherwise can be difficult to obtain due to variations in raw material batches.

For other cereal based beverages, the glucoamylase is also applied for production of malt extract and syrup, where the mashing, separation from spent grain and wort boiling are conducted as described, but the resulting wort is concentrated and optionally spray dried. It can then be used as malt extract or for e.g. other fermentations than beer, other food and beverages, or simply used later for beer fermentation.

During starch processing syrups are made from the starch of different grains. These syrups are used as ingredients in a variety of different beverage and food products. Glucoamylases are especially used for production of glucose syrup. The first step in starch conversion is the liquefaction, where the starch is gelatinised and degraded by amylases into maltodextrin of relatively low viscosity. The maltodextrin is then further degraded in the saccharification step by glucoamylases and sometimes. The glucoamylase is added during saccharification where it degrades starch polysaccharides into glucose.

6.2 Use Levels

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

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

The maximum recommended use level is:

Baking and other cereal based processes

Up to 3200 AGU per kilogram of starch dry matter.

Brewing processes and other cereal based beverage processes

Up to 3000 AGU per kilogram of starch dry matter.

Starch processing

Up to 750 AGU per kilogram of starch dry matter

6.3 Enzyme Residues in the Final Food

The glucoamylase food enzyme is expected to be inactivated or removed during the production process for all applications.

The effect of the glucoamylase is the conversion during food processing of starch (amylose and amylopectin) and hydrolysis products of starch in various foods or food ingredients into D-glucose.

The enzymes exert their function during food processing and do not have any function 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 enzymes during processing, depletion of the substrate, lack of water activity, pH, and removal during processing. All of these conditions would remove any potential residues.

7. SAFETY EVALUATION

7.1 Safety of the Production Organism

The safety of the production organism must be the prime consideration in assessing the degree of safety of an enzyme preparation intended for use in food (2) (1). If the organism is non-toxigenic and non-pathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, is safe to consume (19). Pariza and Foster (2) define a non-toxigenic organism as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a non-pathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances".

Aspergillus niger has a long history of safe use in the production of industrial enzymes and chemicals of both food grade and technical grade. *Aspergillus niger* is listed as a production/donor organism for a series of food-grade carbohydrases, oxidoreductases, lipases, glucanotransferase, and proteases in published scientific literature (1).

Carbohydrase, pectinase, protease, glucose oxidase, catalase, lipase and lactase enzyme preparations from *Aspergillus niger* are included in the GRAS petition 3G0016 (filed April 12th, 1973) that FDA, on request from the Enzyme Technical Association (ETA), converted into separate GRAS Notices (GRN 89, 111, 132) (5). Based on the information provided by ETA, as well as the information in GRP 3G0016 and other information available to FDA, the agency did not question the conclusion that enzyme preparations from *Aspergillus niger* are GRAS under the intended conditions of use. Analogous conclusions were drawn in GRAS Notices GRN 158, 183, 214, 296, 345, 402, 428 which all describe food enzymes produced by *Aspergillus niger* strains (5).

In 1997, *Aspergillus niger* became one of the ten microbial species/strains that were eligible for exemption under 40 CFR Part 725 as recipient microorganisms under the TSCA biotechnology regulations (20). Also, *Aspergillus niger* was reviewed and was concluded to be a safe source organism by Olempska-Beer et al. (21) and Schuster et al. (3) under Good Manufacturing Practice (GMP) and with mycotoxin testing.

An evaluation of the genetically modified production microorganism for the glucoamylase, embodying the concepts initially outlined by Pariza and Foster, 1983 (2) and further developed by IFBC in 1990 (19), the EU SCF in 1991 (9), the OECD in 1992 (10), ILSI Europe Novel Food Task Force in 1996 (13), FAO/WHO in 1996

(12), JECFA in 1998 (18) and Pariza and Johnson in 2001 (1) demonstrates the safety of this genetically modified production microorganism strain. The components of this evaluation: the identity of the recipient strain, a description of the incorporated DNA, the sources and functions of the introduced genetic material, an outline of the genetic construction of the production strain, and some characteristics of the production strain and the enzyme derived from it are given in Section 2 and 3.

Novozymes' used the decision tree (Appendix 2) in Pariza and Johnson 2001 (1) as a basis for our safety assessment. The production strain is genetically modified as discussed in Section 2. The expressed enzyme product is glucoamylase. The enzyme preparation is free of DNA encoding transferable antibiotic resistance DNA genes. The introduced DNA is well characterized and safe for the construction of microorganisms to be used in the production of food grade products. The DNA is stably integrated into the chromosome and the incorporated DNA is known not to encode or express any harmful or toxic substances.

The genetic modifications are well characterized and specific utilizing well-known plasmids for the vector constructs, and the introduced genetic material does not encode and express any known harmful or toxic substances.

Some *Aspergillus niger* strains can produce ochratoxin A (3), and the production of fumonisin B2 has also been shown in *Aspergillus niger* (22). Ochratoxin A and fumonisin B2 are the two mycotoxins of concern in terms of human and animal safety that can be produced by *Aspergillus niger* strains (23).

The BO-1 safe strain lineage was found to be unable to produce unwanted secondary metabolites (ochratoxin A and fumonisin B2) under conditions that are known to induce mycotoxin production in fungi. In addition analytical test results of three representative enzyme batches of this glucoamylase (as discussed in section 5.2) confirm the absence of ochratoxin A and fumonisin B2.

Based on the information presented above it is concluded that the *Aspergillus niger* production strain is considered a safe strain for the production of the glucoamylase enzyme.

7.1.1 Safe Strain Lineage

The *Aspergillus niger* production strains are derived from the *Aspergillus niger* C40 (parental strain) cell lineage. Strain BO-1, derived from the parental strain C40 solely by classical mutagenesis, was deposited as DSM 12665. The identification of strain BO-1 as *Aspergillus niger* has been confirmed and certified by DSMZ. The *Aspergillus niger* production strains belong to a single strain lineage which has been used by Novozymes for production of a wide range of commercial enzymes.

The safety of the *Aspergillus niger* production strain was established following published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients (1) (8).

The recipient strain, *Aspergillus niger ,* has been thoroughly characterized as shown in Sections 2.2. The introduced DNA is well-known and characterized in Section 2.3 and 2.4 and the introduced genetic material does not encode or express any known harmful or toxic substances.

The procedures used to modify the recipient organism are well defined and commonly used. Therefore, the elements needed to establish a safe strain lineage as defined in Pariza and Johnson, 2001 (1) have been met.

Novozymes has extensive experience working with *Aspergillus niger* production strains and has developed expertise in identifying and characterizing these strains in order to prevent contamination and ensure continuing acceptable, economic yields of a functional enzyme product. Research scientists, fermentation engineers, chemical operators, and quality control technicians follow standard aseptic microbiological procedures as well as specific Novozymes protocols for monitoring the biological activity, growth, and physiological characteristics of the production organism during strain improvement programs and during large scale industrial fermentations. In addition, the final commercial enzyme product must perform reproducibly, meet Novozymes' technical service department requirements, and consistently meet the needs of customers in the food industry.

All of these periodic and continuous monitoring activities serve not only to guarantee customer satisfaction with Novozymes' enzyme products but also indicate that no unexpected secondary effects of the genetic modifications have been observed. Furthermore the information included in this notification has been reviewed by Novozymes Regulatory Affairs staff for suitability.

Novozymes has used *Aspergillus niger* production strains derived from the BO-1 lineage for over 20 years and has performed a number of safety studies on different enzyme products manufactured using *Aspergillus niger* . Table 3 below outlines some of the Novozymes products produced by *Aspergillus niger* strains and the safety studies conducted on those products. Section 7.5.1 outlines the testing completed and the appendix reference.

Table 3. Safe strain lineage

Enzyme	EC No.	Predecessor strain (a)	Donor strain	Safety studies (b)
Glucoamylase	3.2.1.3	*Aspergillus niger* BO-1	None	Yes
Pectin lyase	4.2.2.10	*Aspergillus niger* BO-1	*Aspergillus niger*	Yes
Lysophospholipase	3.1.1.5	*Aspergillus niger* BO-1	*Aspergillus niger*	Yes
Triacylglycerol lipase	3.1.1.3	*Aspergillus niger* BO-1	*Candida antarctica*	Yes

7.3.1 Allergenic Potential of the Glucoamylase 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) (30).

In order to further evaluate the possibility that the glucoamylase 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 (31) and modified by Codex Alimentarius Commission, 2009 (32) the glucoamylase 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**).

The following homology analyses were conducted:

1. More than 35% identity in the amino acid sequence of the expressed protein (i.e. without the leader sequence, if any), using a window of 80 amino acids and a suitable gap penalty (using Clustal-type alignment programs or equivalent alignment programs).

2. Same as item 1, but with scaling enabled. In this way matches with high identity, but over windows shorter than 80 amino acids, can be identified.

3. Alignment of relevant protein (enzyme) to each of the allergens and identifying hits with more than 35% identity over the full length of the alignment.

4. Also, a search for 100% identity over 8 contiguous amino acids was completed.

Results and discussion:

Allergen analysis of 1,4-alpha-D-glucan glucohydrolase from Penicillium oxalicum, using the database, identified one fungal allergen having an identity with 1,4-alpha-D-glucan glucohydrolase from Penicillium oxalicum significantly above the threshold of 35%.

The 1,4-alpha-D-glucan glucohydrolase Sch c 1 of Schizophyllum commune was revealed to have 65.0% hence 69.5% (with and without scaling) identity over 80 amino acids windows using the allergenonline, database.

Similarities with other allergens were below 35%. Since it is generally accepted that proteins with such a low identity rarely share epitopes they were not considered.

There is compelling evidence that the vast majority of adults 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 (33).

To our knowledge, there is no evidence suggesting that 1,4-alpha-D-glucan glucohydrolase Sch c 1 triggers oral sensitization.

This is backed up by the study conducted by Bindslev-Jensen et al (30) using the generally recognized guidelines for food allergy diagnosis (skin prick test, specific serum IgE and DBPCFC).

Conclusion:

On the basis of the available evidence as reported in Appendix 4, it is concluded that oral intake of glucoamylase produced by *Aspergillus niger* strain is not anticipated to pose any food allergenic concern.

7.4 Safety of the Manufacturing Process

The glucoamylase enzyme preparation meets the purity criteria for enzyme preparations as outlined in the monograph on Enzyme Preparations in the *Food Chemicals Codex.* As described in Section 3, the enzyme preparation is produced in accordance with current good manufacturing practices, using ingredients that are acceptable for general use in foods, and under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for production of microbial enzymes (14) (15) (16).

7.5 Safety studies

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

7.5.1 Description of Test Material

The following studies were performed on test batch PPY34422 with favourable results:

- Bacterial Reverse Mutation Assay (Ames test)
- *In Vitro* Micronucleus Assay
- 13 Week Oral Toxicity Study in Rats

These tests are described in Appendix 3.

7.6 Estimates of Human Consumption and Safety Margin

The exposure assessment is performed according to the Budget Method (34) (35) (36). Overall, the human exposure to the glucoamylase will be negligible because the enzyme preparation is used as a processing aid at lower dosages.

The Budget Method assumptions represent a "maximum worst case" situation of human consumption, in which the food enzyme, the object of this notification, would be used at its maximum recommended dosages in all processed food and all processed beverages and not only in those food and drink processes described in Section 6.1 and Section 6.2.

It is also supposed that the totality of the food enzyme will end up in the final food. This assumption is exaggerated since the enzyme protein and the other substances resulting from the fermentation are diluted or removed in certain processing steps. Therefore the safety margin calculation derived from this method is highly conservative.

Assumptions in the Budget Method

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

Liquids: The maximum intake of liquids (other than milk) is 100 ml/kg body weight (bw) day. Assuming that 25% of the non-milk beverages 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% hydrolyzed starch = 2.50 g starch derived dry matter per kg body weight per day.

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

TMDI calculation

7.6.2 Safety margin

Solid Food

The highest dosage given in Section 6.2 for solid food is 3200 AGU per kg starch based raw material. 3200 AGU correspond to 2223 mg TOS.

Based on this, 3.12 gram starch-derived dry matter in solid food will maximally contain: 2223 mg TOS per kg / 1000 g per kg x 3.12 g = 6.94 mg TOS

Liquid Food

The highest dosage given in section 6.2 for liquid food is 3000 AGU per kg starch based raw material. 3000 AGU corresponds to 2084 mg TOS.

Based on this, 2.50 gram starch-derived dry matter in liquids will maximally contain: 2084 mg TOS per kg / 1000 g per kg x 2.50 g = 5.21 mg TOS

The theoretical maximum daily intake (TMDI) of consumers of the food enzyme is: 6.94 + 5.21 = 12.15 mg TOS/kg body weight/day

Calculation of Safety Margin

The NOAEL in the 13 weeks oral toxicity study in rats was concluded to be 1360 mg TOS/kg body weight/day).

Based on the calculated theoretical "worst case" TMDI as described above a safety margin can be calculated as the dose level with NOAEL divided by the TMDI, as seen below.

Table 4. NOAEL Calculation

NOAEL (mg TOS/kg bw/day)	1360
*TMDI (mg TOS/kg bw/day)	12.15
Safety margin	112

*based on the worst case scenario

8. Results and Conclusions

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

9. List of Appendices

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

2. Pariza and Johnson Decision Tree Analysis

3. Summary of Toxicity Data. November 2013, LUNA No. 2013-14741.

4. Allergen Risk Assessment Report. May 2016, LUNA No. 2016-08301.

10. 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.

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

3. Schuster, E.; Dunn-Coleman, N.; Frisvad, J.C.; van Dijck, P.W.M. On the safety of Aspergillus niger - a review. Appl. Microbiol. biotechnol. 59, 426-435. 2002.

4. Environmental Protection Agency (EPA). Biotechnology Program Under Toxic Substances Control Act (TSCA) Bacillus licheniformis Final Risk Assessment 1997. (http://epa.gov/oppt/biotech/pubs/fra/fra005.htm).

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

6. US Environmental Protection Agency. Final risk assessment and decision document to exempt A. niger strains from the TSCA Biotechnology Program, 1997.

7. Association of manufacturers and formulators of enzyme products (Amfep). List of enzymes. Amfep/09/68, www.amfep.org/content/list-enzymes, 2009.

8. 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.

9. EU Scientific Committee for Food. Guidelines for the presentation of data on food enzymes. Reports of the Scientific Committee for Food, 27th series, 1991.

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

11. Berkowitz, D. and Maryanski, J.. Implications of biotechnology on international food standards and codes of practice. Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission, Eighteenth Session, Geneva, July 3-12, 1989.

12. FAO/WHO. Biotechnology and Food Safety, Report of a Joint FAO/WHO Consultation. FAO Food and Nutrition Paper 61. Rome, Italy. 1996.

13. Jonas, D.A., et el. The Safety Assessment of Novel Foods, Guidelines prepared by ILSI Europe Novel Food Task Force, Food Chemical Toxicology, Vol. 34, 1996. pp. 931-940.

14. 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.

15. 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.

16. 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.

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

18. 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).

19. 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.

20. US Environmental Protection Agency. Final risk assessment and decision document to exempt A. niger strains from the TSCA Biotechnology Program.

21. 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.

22. Frisvad JC, Smedsgaard J, Samson RA, Larsen TO, Thrane U. Fumonisin B2 Production by Aspergillus niger. J. Agric. Food Chem. 55, 9727–9732. 2007.

23. Nielsen KF, Mogensen JM, Johansen M, Larsen TO, Frisvad JC. Review of secondary metabolites and mycotoxins from the Aspergillus niger group. Anal Bioanal Chem, 395, 1225–1242. 2009.

24. Food and Drug Administration. Lipase Enzyme Preparation From Rhizopus niveus: Affirmation of GRAS status as a Direct Food Ingredient. Fed. Regist. 63:24416-24419, 1998.

25. Food and Drug Administration. Statement of Policy: Foods Derived From New Plant Varieties. Fed. Regist. 57:22984-23005, 1992.

26. Food and Drug Administration. Enzyme Preparations from animal and plant sources; Affirmation of GRAS status as direct food ingredients. Fed. Regist. 60, 1995. pp. 32904-32912.

27. Godfrey, T. Brewing. Industrial Enzymology. The application of enzymes in industry (Godfrey T, Reichelt J eds.) The Nature Press, New York, 221-259, 1983.

28. Reichelt, JR. Starch. Industrial Enzymology. The application of enzymes in industry (Godfrey T, Reichelt J eds.) The Nature Press, New York, 375-396,1983.

29. van Oort, M. Enzymes in bread making. Enzymes in Food Technology (RJ Whitehurst, M van Oort M, eds) Wiley-Blackwell, UK. 103-143, 2010.

30. 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.

31. 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.

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

33. Brisman J., Baker's Asthma. Occup. Environ. Med. 59, 498-502, 2002.

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

35. Douglass, J.S., Barraj, L.M., Tennant, D.R., Wesley, R.L., Chaisson, C.F. Evaluation of the Budget Method for screening food additive intakes. Food Additives and Contaminants. 14 (8), 791-802, 1997. .

36. ILSI Europe Food Chemical Intake Task Force. An Evaluation of the Budget Method for Screening Food Additive Intake. April 1997.

Toxicology & Product Safety

Date : 13. November 2013
File : 2013-14741
Ref.: TrGQ/PBjP/LiNi

SUMMARY OF TOXICITY DATA

Glucoamylase, batch PPY34422 from *Aspergillus niger*

Authors:
Trine Østergaard Lund
Peder Bjarne Pedersen
Line Anker Nielsen

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

C O N T E N T S

PAGE

1. ABSTRACT

The below series of toxicological studies were undertaken to evaluate the safety of Glucoamylase, batch PPY34422.

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 Novozymes A/S, Denmark, Covance Laboratories, England and at Huntingdon Life Sciences, England during the period November 2012 to August 2013.

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

- Glucoamylase, batch PPY34422 did not induce gene mutations in the Ames test, neither in the presence or absence of S-9 mix.
- Glucoamylase, batch PPY34422 did not show any clastogenic activity, neither in the presence or absence of S-9 mix, when tested in the *in vitro* micronucleus assay.
- Daily oral administration (by gavage) of Glucoamylase, batch PPY34422 to rats at dosages of up to 10.0 mL/kg bw/day for thirteen weeks resulted in no treatment-related effects. Consequently, the No Observed Adverse Effect level (NOAEL) was considered to be 10 mL/kg/day (equivalent to 1612 AMGU(D)/kg bw/day or 1.36 g TOS/kg/ day).

Based on the present toxicity data and the fact that Glucoamylases have a history of safe use, it is our conclusion that Glucoamylase represented by batch PPY34422, can be considered as generally safe.

2. TEST SUBSTANCE

Glucoamylase is a liquid enzyme concentrate containing a T-stable glucoamylase (E.C. number 3.2.1.3).

2.1 Production Organism

Glucoamylase is produced by a strain of *Aspergillus niger.* It contains the gene of a protein engineered variant of a glycoamylase obtained from a strain of *Penicillum oxalicum*.

A. niger has long history of safe use and it 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. It is accepted as well as the enzymes it produces as a constituent of food (FAO/WHO JECFA, 1987) and the recombinant production organisms comply with OECD recommendations on Good Industrial Large Scale Practice organisms (OECD, 1986).

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 development of the production strain was evaluated at every step to assess incorporation of the desired functional genetic information and to ensure that no unintended sequences were incorporated.

A few percent of wild type strains of *A.niger* may have the potential to produce the mycotoxins ochratoxin and/or fumonisin B_2. The metabolite profile of the strain lineage to which the production strain belongs has been investigated and the potential to produce these mycotoxins was not demonstrated. Additionally, Glucoamylase, batch PPY34422, has been analysed for the presence of ochratoxin as well as Fumonicin B_2 and none of these mycotoxins were detected.

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

2.2 Characterization

One batch, PPY34422, was used for the conduct of the toxicological studies. The characterization of the batch is presented in Table 1.

Table 1.
Characterization data of Glucoamylase, batch PPY34422

Batch number	PPY34422
Activity in AGU	187
Activity in AMGU(D)/g	154
Water (% w/w)	(KF) 86.7
Dry matter (% w/w)	13.3
Ash (% w/w)	0.3
Total Organic Solids (TOS[1]) (% w/w)	13.0
Specific gravity (g/mL)	1.047

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

3. MUTAGENICITY

3.1 Bacterial Reverse Mutation Assay (Ames test)

Glucoamylase, batch PPY34422 was examined for mutagenic activity in the bacterial reverse mutation assay using *Salmonella typhimurium* strain TA1535, TA100, TA1537, and TA98 and *Escherichia coli* WP2uvrA. The study was carried out according to the OECD test guideline 471 (adopted in 1997) and in compliance with GLP.

Crude enzyme preparations, like the present test compound, contain the free amino acids histidine and tryptophan, most often in an amount, which exceeds the critical concentration for incorporation in the direct standard assay.
To overcome this problem all strains were exposed to Glucoamylase, batch PPY34422 in liquid culture ("treat and plate assay").

Bacteria were exposed to 6 doses of the test substance in a phosphate buffered nutrient broth for 3 with 5000 µg (dry matter)/ml as the highest concentration. After incubation the test substance was removed by centrifugation prior to plating.

The study was conducted with and without the metabolic activation system S9 – a liver preparation from male rats, pre-treated with Aroclor 1254, and the co-factors required for mixed function oxidase activity (S9 mix). All results were confirmed by conduction of two complete and independent experiments.

Glucoamylase, batch PPY34422 is a fluid enzyme preparation. It contains an abundance of various nutrients, and composes a rich growth medium to the test bacteria. This may cause some fluctuation in the number of spontaneous revertant colonies, which is to some degree reflected in the present study.

The test substance was not toxic to any of the bacterial strains no reductions in the growth of the background lawn of the non-revertant bacteria were observed at any dose level.

No treatments of any of the *Salmonella* and *E. coli* strains with Glucoamylase, batch PPY34422 resulted in any increases in the number of revertant colonies that meet the criteria for a positive or equivocal response.

Based on the results obtained, it is concluded that Glucoamylase, batch PPY34422 did not show evidence of mutagenic activity when tested under the conditions applied in this study.

3.2 *In Vitro* Micronucleus Assay

In order to assess the clastogenic and the aneugenic activity of Glucoamylase, batch PPY34422, its effects on the frequency of micronuclei was investigated in cultured human peripheral blood lymphocytes applying the cytokinesis-block methodology.

The study was conducted according to GLP, in compliance with the OECD test guideline 487 (adopted in 2010).

Heparinized whole blood cultures, pooled from two female donors, were established, and division of the lymphocytes was stimulated by adding phytohaemagglutinin (PHA) to the cultures.

Two independent experiments were performed. Sets of duplicate cultures were treated with the solvent (purified water), test substance or appropriate positive controls. Treatments with the test substance covered a broad range of doses, separated by narrow intervals. The highest concentrations used was 5000 µg/mL (expressed in terms of the test substance as supplied), which is the highest dose level recommended in the guidelines for *in vitro* cytogenetic assays.

Sets of duplicate cultures were exposed to the test substance for 3 hours in the presence and absence of metabolic activation (S-9 mix) and harvested 24 hours after the beginning of treatment (3+21 hour treatment). Additionally, a continuous 24-hour treatment without S-9 mix was included with harvesting 24 hours after removal og the test substance (24+24 hour treatment). The cultures were treated with cytochalasin-B after removal of the test substance. Three concentrations, covering an appropriate range of cytotoxicity, were selected for scoring of micronuclei by evaluating the effect of the test substance on the replication Index (RI). 2000 cells per concentration (1000 cells from each replicate culture) were scored.

The proportion of binucleate cells with micronuclei in all cultures of the vehicle controls (purified water) was within the limits of the historical ranges. The positive controls induced statistically significant increases in the proportion of cells with micronuclei, thus demonstrating the sensitivity of the test procedure and the metabolic activity of the S-9 mix employed.

Treatment of the cells with the test substance resulted in frequencies of micronucleated binuclear cells (MNBN cells), which were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle controls for the majority of concentrations analysed. An exeption to this was noted for the 24+24 hour -S-9 treatment at the highest concentration analysed (5000 µg/mL), where a small but statistically significant increase was seen. However, this statistically significant increase was small such that both replicate cultures at this and all other test article concentrations analysed fell within normal ranges with no evidence of a concentration related effect. As such, this small statistical increase was not considered of biological importance. Following the 3+21 hour + S-9 treatment, a single replicate (replicate B) culture at the highest concentration analysed (5000 µg/mL) fell above normal ranges. Further analysis of additional cells from non-scored slides from both the A and B replicate cultures at this concentration, demonstrated an overall response that fell within normal values. As such, this isolated observation was considered spurious and of no biological importance.

It was concluded that Glucoamylase, batch PPY34422 did not induce micronuclei in cultured human peripheral blood lymphocytes either in the absence or presence of S-9 mix under the experimental conditions employed for this study.

4. GENERAL TOXICITY

4.1 13 Week Oral Toxicity Study in Rats

Groups of 10 male and 10 female Spraque-Dawley rats were dosed Glucoamylase, batch PPY34422 orally by gavage at dosages of 1.0, 3.3 or 10.0 mL/kg/day (equivalent to 0.136, 0.449 or 1.36 g TOS/kg/day or 161, 532 or 1612 AMGU(D)/kg/day). A similarly constituted control group received the vehicle (reverse osmosis water) at the same volume-dose (10 mL/kg bodyweight).

During the study, clinical condition, detailed physical examination and arena observations, sensory reactivity, grip strength, motor activity, bodyweight, food consumption, ophthalmic examination, haematology, blood chemistry, organ weight, macropathology and histopathology investigations were undertaken.

There were no deaths during the treatment period.

At the motor activity assessment, minor but statistically significant reductions in scores were recorded for the high dose males for cage floor activity during the second and seventh six minute intervals but because of the isolated nature of these differences, and the absence of similar trends in females, this finding was considered to reflect normal variation.

Slightly extended activated partial thromboplastin times were seen in animals receiving 10 mL/kg/day but this change was considered unrelated to treatment and of no toxicological importance because the differences from control were < 2 seconds and all individual values were within the background range of historical data. In females, a small reduction in prothrombin time was observed but all values were within the historical data range and hence, no association with treatment was inferred.

The blood chemistry investigation revealed a statistically significant slight increase in plasma potassium concentration in females receiving 10 mL/kg of the test compound. The majority of high dose females had potassium concentrations that were above the range reported for the contemporary controls but all were within the historical data range and there was no similar trend in males. This change was therefore considered fortuitous and not related to treatment.

A reduction of liver weight was seen at all doses in females, when compared with the controls. This was not attributed to treatment because the differences from controls were minimal, lacked dose-relationship and there was no similar trend in the males. Moreover, the histopathological examination of the livers from the high dose females did not reveal any lesions associated with the administration of Glucoamylase, PPY34422 and none of the livers in the mid and low dose groups appeared abnormal at necropsy.

There were no treatment-related ophthalmoscopic findings and no macroscopic or microscopic findings that were attributable to treatment

It is thus concluded that oral administration of Glucoamylase, batch PPY34422 to Sprague-Dawley [Crl:CD(SD)] rats at doses up to 100% of the test compound (equivalent to 1.36 g TOS/kg/day or 1612 AMGU(D)/kg/day) was well tolerated and did not result in any treatment-related or toxicologically significant change. The no-observed-adverse-effect-level (NOAEL) was therefore greater than 1.36 g TOS/kg/day or 1612 AMGU(D)/kg/day.

5. CONCLUSION

Based on the present toxicity data and the fact that Glucoamylases have a history of safe use, it is our conclusion that Glucoamylase represented by batch, PPY34422, can be considered as generally safe.

6. REFERENCES

6.1 Study Reports

Novozymes A/S: Study No.: 20128083. Glucoamylase PPY34422: Test for mutagenic activity with strains of *Salmonella typhimurium* and *Escherichia coli.* (February 2013). LUNA file: 2013-00438.

Covance Laboratories: Study No.: 8279843. Novozymes Reference No.: 20136013: Glucoamylase, PPY34422: Induction of micronuclei in cultured human peripheral blood lymphocytes. (May 2013). LUNA file: 2013-07350.

Huntingdon Life Sciences: Study No.: LKG0077. Novozymes Reference No.: 20136008: Glucoamylase, PPY34422: Toxicity Study by Oral Gavage Administration to Sprague-Dawley Rats for 13 Weeks. LUNA file: 2013-14731.

6.2 Guidelines

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.

Appendix 3- Pariza & Johnson Decision Tree analysis of a glucoamylase produced by a genetically modified strain of *Aspergillus niger*.

This glucoamylase produced by a genetically modified strain of *Aspergillus niger* was evaluated according to the decision tree published in Pariza and Johnson, 2001.

The result of the evaluation is presented below.

Decision Tree

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

   The production strain *Aspergillus niger* designated 126-PE001-1467-1, was derived via the recipient strain M1371 a natural isolate of *Aspergillus niger* strain BO-1.
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
      Enzymes, including glucoamylase, have a long history of use in food and animal feed. *Aspergillus niger* has been used by industry since 1919 for the production of citric acid which could be an ingredient of foods such as soft drinks, fruit juices and jams. The US Food and Drug Administration (FDA) listed *Aspergillus niger* as a source of citric acid (21 CFR 173.280). The glucoamylase which is the subject of this GRN, is produced by a production strain that is from a safe strain lineage with an extended history of safe use
      If yes go to 3c. If no, go to 3b
   b. Is the NOAEL for the test article in the appropriate short-term oral studies sufficiently high to ensure safety? (Not required since 3a is YES however the answer here is also **YES**)

   c. Is the test article free of transferable antibiotic resistance gene DNA?

      YES

      No functional antibiotic resistance genes were left in the strain as a result of the genetic modifications. The absence of these genes was verified.
      If yes go to 3e. If no go to 3d.

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
The genetic modifications are well characterized and specific and the incorporated DNA does not encode and express any known harmful or toxic substances.
If yes, go to 4.

4. Is the introduced DNA randomly integrated into the chromosome?
NO
Site specific integration of the DNA was achieved at several selected loci of the *Aspergillus niger* chromosome. Sequence confirmation was performed in the production strain.
If yes go to 5. If no, go to 6.

5. Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed?
YES
If yes go to 6. If no go to 7.

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

The *Aspergillus niger* production strains are derived from the *Aspergillus niger* C40 (parental strain) cell lineage. Strain BO-1, derived from the parental strain C40 solely by classical mutagenesis, was deposited as DSM 12665. The identification of strain BO-1 as *Aspergillus niger* has been confirmed and certified by DSMZ. The *Aspergillus niger* production strains belong to a single strain lineage which has been used by Novozymes for production of a wide range of commercial enzymes.

The safety of the *Aspergillus niger* production strain was established following published criteria for the assessment of the safe use of microorganisms used in the manufacture of food Novozymes has used *Aspergillus niger* production strains derived from the BO-1 lineage for over 20 years and has performed a number of safety studies on different enzyme products manufactured using *Aspergillus niger*.

If yes the test article is ACCEPTED.

novozymes®
Rethink Tomorrow

Toxicity and Allergenicity Risk Assessment Report

By ELRo, EPF

Gluco-amylase (amyloglucosidase) PPY34422 from Aspergillus niger/ Penicillum oxalicum/Bf-3

Toxicity assessment:

The homology between gluco-amylase PPY34422 and known toxins was assessed on the basis of the information present in the UNIPROT database (24-Jan-2013). This database contains entries from SWISSPROT and TREMBL. The homology among the emerging entries did not exceed 20% indicating that the homology to any toxin sequence in this database is random.

Assessment of sequence homology to known allergens

Assessment of homology of the amino acid sequence of gluco-amylase PPY34422 to known allergens according to the EFSA's Scientific opinion[1] using the databases allergenonline.org and allergen.org, identified one fungal allergen having an identity with gluco-amylase PPY34422 significantly above the threshold of 35%.

The gluco-amylase Sch c 1 of *Schizophyllum commune* was revealed to have 60-84% identity with gluco-amylase PPY34422 over 80 amino acids windows (with and without scaling) using the allergen.org database.

Similarities with other allergens were below 35%. Since it is generally accepted that proteins with such a low identity rarely share epitopes they were not considered in this report (Pearson, 2000; Aalberse et al., 2001; Hileman et al., 2002; Ladics et al., 2007).

Risk assessment:

Like fungal α-amylases (prevalence: 23%), fungal-derived gluco-amylases also have been reported to trigger occupational respiratory sensitization and asthma in bakers (prevalence: 8%) (Sander et al., 1998; Quirce et al., 2002).

[1] EFSA (2010) Scientific Opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed. EFSA Journal 8 (7), 1700, 1-168. http://www.efsa.europa.eu/en/efsajournal/doc/1700.pdf. (Last visited 6 February 2013)

Respiratory allergy:

The analysis suggests similarities between gluco-amylase PPY34422 and a well-known respiratory allergen. The risk for respiratory sensitization and for acquiring asthma associated with occupational handling of enzymes has been recognized for many years, and has resulted in clearly described measures for assuring occupational safety.

Food allergy:

There is compelling evidence that vast majority of adults 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 (Brisman, 2002; Armentia et al., 2009).

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. Both studies concluded that no cases of IgE-mediated food allergy to commercial enzymes (incl. gluco-amylase) could be found. There were further 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 (Bindslev-Jensen et al., 2006).

Conclusion:

On the basis of the available evidence it can be concluded that oral intake of gluco-amylase PPY34422 is not anticipated to pose any food allergenic concern, and that the current measures for assuring occupational safety are sufficient.

References:

- Aalberse, R., C., Akkerdaas, J., van Ree, R. 2001. Allergy 56; 478-490
- Armentia, A., Díaz_Perales, A., Castrodeza, J., Dueñas-Laita, A., Palacin, A., Fernández S. 2009. Allergol. Immunopathol. 37; 203-204
- Bindslev-Jensen, C., Skov, P., S., Roggen, E., L., Hvass, P., Brinch, D., S. 2006. Food and Chem. Toxicol. 44; 1909-1915.
- Brisman, J. 2002. Occup. Environ. Med. 59; 498-502.
- Hileman, R., E., Silvanovitch, A., Goodman, R., E., Rice, E., A., Holleschak, G., Ashwood, J.,D., Hefle, S., L. 2002. Int. Arch. Allergy Immunol. 128; 280-291.
- Ladics, G., S., Bannon, G., A., Silvanovitch, A., Cressman, R., F. 2007. Mol. Nutr. Food Res. 51; 985-998
- Pearson, W., R. 2000. Methods Mol. Biol. 132; 185-219
- Sander, I., Raulf-Heimsoth, M., Siethoff, C., Lohaus, C., Meyer, H., E., Baur, X. 1998. J. Allergy Clin. Immunol. 102; 256-264.
- Quirce, S., Fernández-Nieto, M., Bartolomé, B., Bombín, C., Cuevas, M., Sastre, J. 2002. Ann. Allergy Asthma Immunol. 89; 197-202.

SUBMISSION END