A Trehalase Enzyme from *Myceliophthora sepedonium* Produced in *Aspergillus niger*

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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 trehalase enzyme preparation, produced by submerged fermentation of a genetically modified *Aspergillus niger* microorganism carrying the gene coding for trehalase from *Myceliophthora sepedonium.*

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

Classification:	hydrolases
IUBMB nomenclature:	alpha, alpha-trehalase
EC No.:	3.2.1.28
CAS No.:	9025-52-9
Specificity:	catalyzes the hydrolysis of trehalose
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 *Aspergillus niger* production strain, designated C3085-1850-19, was derived via the recipient strain, C3085, from 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 (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 expression plasmid, used in the strain construction, pHUda1850, contains strictly defined chromosomal DNA fragments and synthetic DNA linker sequences. The DNA sequence for the introduced gene is based on the *treMS* sequence encoding a trehalase from *Myceliophthora sepedonium*.

2.2(b) Recipient Strain

The recipient strain C3085 used in the construction of the *Aspergillus niger* production strain was modified at several chromosomal loci during strain development to inactivate genes encoding a number of amylases and proteases amongst others. Furthermore, the fumonisin gene cluster and the oxaloacetate hydrolase gene were deleted in C3085 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.

2.2(c) Trehalase Expression Plasmid

The expression plasmid, pHUda1850, used to introduce the *treMS* gene in the recipient strain C3085 is based on the replication origin of *E. coli*. However, no fragments of the vector backbone are introduced into the production strain. The introduced DNA consists of a fragment of the *Aspergillus niger* promotor, the *treMS* sequence encoding the trehalase, a transcriptional terminator and finally a selective marker; *amdS* encoding an acetamidase.

The expression cassette and the *amdS* 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.

2.2(d) Construction of the Recombinant Microorganism

The production strain, *Aspergillus niger* C3085-1850-19, was constructed from the recipient strain C3085 through the following steps:

1) Plasmid pHUda1850 was integrated into two specific loci in strain C3085 by targeted homologous recombination to these loci. Targeted integration of the expression cassettes at these loci allows the expression of the *treMS* gene from the promoter.

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

The trehalase production strain containing one *treMS* gene at each of the two target loci was named C3085-1850-19.

Sequence confirmation of the inserted expression cassettes and the flanking regions at each of the integration loci 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. 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.

2.2(f) Antibiotic Resistance Gene

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

2.2(g) Absence of Production Organism in Product

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

2.3 METHOD OF MANUFACTURE

This section describes the manufacturing process for the trehalase enzyme which follows standard industry practices (9) (10) (11). The quality management system used in the manufacturing process for the enzyme preparation 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. These methods are based on generally available and accepted methods used for production of microbial enzymes.

The enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in the Food Chemicals Codex (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 enzyme concentrate are standard ingredients used in the enzyme industry (9) (10) (11). 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 trehalase enzyme preparation is produced by pure culture submerged fed-batch fermentation of a genetically modified strain of *Aspergillus niger* as described in Part 2. All equipment is carefully designed, constructed, operated, cleaned, and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are done to ensure absence of foreign microorganisms and confirm strain identity.

2.3(c) Production Organism

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

2.3(d) Criteria for the Rejection of Fermentation Batches

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

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

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

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

Any contaminated fermentation is rejected.

2.3(e) Recovery Process

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

2.3(f) Purification Process

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

1) Pretreatment - pH adjustment and flocculation (if required)

2) Primary Separation – vacuum drum filtration or centrifugation

3) Concentration - ultrafiltration and/or evaporation

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

5) Final concentration – evaporation and/or ultrafiltration.

6) Preservation and stabilization of the liquid enzyme concentrate

The enzyme concentrate is stabilized with glycerol. The liquid product is formulated by addition of water and preserved with potassium sorbate, sodium bisulphite and sodium benzoate. See Table 1 below.

2.4 COMPOSITION AND SPECIFICATIONS

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

2.4(a) Quantitative Composition

The trehalase enzyme preparation is sold in a liquid form. Table 1 below identifies the substances that are considered diluents, stabilizers, preservatives and inert raw materials used in the enzyme preparations. The enzyme preparation does not contain any major food allergens from the fermentation media.

Table 1. Typical compositions of the enzyme preparations

Substance	Approximate Percentage
Enzyme Solids (TOS*)	13%
Water	40 - 50%
Glycerol	25-50%
Sodium Bisulfite	<0.5%
Sodium Benzoate	<0.5%
Potassium Sorbate	<0.5%

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

2.4(b) Specifications

The trehalase enzyme preparation complies with the recommended purity specification criteria for "Enzyme Preparations" as described in *Food Chemicals Codex* (12). 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 in Table 2 below.

Table 2. Analytical data for three food enzyme batches

Parameter	Specifications	PPT42569	PPT42633	PPT43293
Trehalase activity	TNU(A)/g	1310	1220	1140
Total viable count	Upper limit 50,000	<100	<100	<100
Lead	Not more than 5 mg/kg	<0.5	<0.5	<0.5
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
Ochratoxin A	<LOD mg/kg	<0.0003	<0.0003	<0.0003
Fumonisin B2	<LOD mg/kg	<0.0003	<0.0003	<0.0003

2.5 PHYSICAL OR TECHNICAL EFFECT

2.5(a) Mode of Action

The active enzyme, and the subject of this notification, is trehalase (EC 3.2.1.28).

Trehalase enzymatically cleaves the disaccharide trehalose into two glucose molecules.

In the process of alcohol production, the starch contained in grain raw materials is extracted or made accessible by finely grinding and precooking the grain. Trehalase is then added to the grain slurry in order to hydrolyze trehalose into glucose molecules. The glucose is then used by the yeast as a source of food during fermentation. The ethanol is subsequently concentrated and recovered by distillation.

Trehalase enzymes are advantageous in ethanol processing as it increases glucose accessibility via it's enzymatic action on trehalose.

Enzymes, such as trehalase, added to the grain slurry either during starch hydrolysis or directly into the fermenter, are for purposes such as: to facilitate the degradation of starch and non-starch polysaccharides into fermentable sugars, improve ethanol yield and allow the use of higher process temperatures and lower pH in order to enhance process efficiency.

2.5(b) Use Levels

The trehalase enzyme preparation will be added to grain slurry at levels no higher than necessary to achieve an intended 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 recommended use level is 0.0184 TNU(A) per gram dry solids.

2.5(c) Enzymes Residues in the Final Food

Once the enzyme preparation is added, the grain slurry preparation must still be processed through a fermentation step before entering a distillation column for separation of the distillate and the distiller's grains.

Each of these products, as a function of ethanol production, will be exposed to temperatures in excess of 80°C, resulting in the thermal inactivation or reduction to negligible or undetectable levels of the enzyme. The fermentation step and distillation step involve aggressive processing including high temperatures and low pH levels, both of which are not conducive to maintaining the structure of the enzyme protein. The aggressive processing will also nearly sterilize the stillage. These changes will denature the enzyme protein once it has completed the glucose conversion.

Therefore, the enzyme will not be present or active in the final food/beverage.

PART 3 - DIETARY EXPOSURE

In the distillation process, non-volatile substances such as enzymes will not distill. Consequently, there will be no dietary exposure of enzymes used in distillation processes as further outlined below.

Distillation and rectification allows the concentration of alcohol to be increased by separating ethanol from water and other impurities in the mash. Ethanol is recovered from the mash by a multistage continuous counter current distillation ("continuous rectification"). Using heat, ethanol and other volatile substances are evaporated from the feed mash in a column. At the top of the column, the lower volatile substances are condensed and returned as liquid in the column. The more volatile substances pass into a second column, where the process is repeated, but with already highly purified ethanol. In both columns the reflux liquid would return non-volatile substances or those with a lower vapor pressure than ethanol, to the bottom of the respective column.

Non-volatile substances are those substances that have no relevant vapor pressure which is typically the case for solids. Through this process, non-volatile substances will not pass into the ethanol. Enzymes are macromolecular proteins that accelerate biochemical reactions. They are consequently non-volatile substances, with no vapor pressure.
In order to further substantiate this, we refer to the index of monographs on compounds identified in alcoholic beverages, published by the World Health Organization's IRAC (International Agency for Research on Cancer) committee, (14). These monographs provide a qualitative and quantitative overview on the substances that are present in alcoholic beverages. Based on the information provided by the monograph's molecular formulas for these compounds it is clear that most of the substances occurring in distilled beverages have a molecular weight below 600 Da, making them volatile. The molecular weight of enzymes varies from around 10,000 Da to 1,000,000 Da (15). Thus, providing further evidence that enzymes are considered non-volatile substances and will not distill.

Additionally, the European Food Safety Authority (EFSA) has assessed residual amount of proteins originating from cereal grains, whey and nuts in distilled beverages. Enzyme proteins exhibit the same physical characteristics as these proteins and the results are therefore comparable.

The conclusions of EFSA were published in the EFSA Journal in the form of three separate opinions (16) (17) (18) Based on the evidence provided, the EFSA Panel noted that proteins and peptides "are not carried over into distillate during a properly controlled distillation process" at least not in amounts greater than 1 mg/l for total protein.

Even if all the protein remaining in the distillate is enzyme protein this means that only 1mg/L of enzyme protein remains in the alcohol. This equates to 10 ppm, which is

negligible. For all practical purposes, the enzyme TOS is eliminated by the distillation step.

3(a) Assumptions in Dietary Exposure

Based on the explanation above, this section is not applicable.

3(b) Food Consumption Data

Based on the explanation above, this section is not applicable.

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 trehalase 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.

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 (4) (5) (1) (6) (7) (8). The methods used to develop the genetically modified production organism and the specific genetic modifications introduced into the production organism are described in Part 2.

6(a) Safety of the Production Organism

The safety of the 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 trehalase, Aspergillus niger, is discussed in Part 2 and also in this Part.

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 (3)

If the organism is non-toxigenic and non-pathogenic, then it is assumed that foods or food ingredients produced from the organism, using current Good Manufacturing Practices, are safe to consume (19). Pariza and Foster 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".

Some Aspergillus niger strains can produce ochratoxin A (20). And, the production of fumonisin B2 has also been shown in Aspergillus niger (21). 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 (22).

The BO-1 safe strain lineages were 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 trehalase confirm the absence of ochratoxin A and fumonisin B2. See Table 2.

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 (23). Also, Aspergillus niger was reviewed and was concluded to be a safe source organism by Olempska-Beer et al (24) and Schuster et al (20) under Good Manufacturing Practice (GMP) and with mycotoxin testing.

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) (25). 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 (25).

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. Novozymes has used Aspergillus niger production strains derived from the C40 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.

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
Glucoamylase	3.2.1.3	*Aspergillus niger* JaL303	*Aspergillus niger*	Yes
Glucoamylase	3.2.1.3	*Aspergillus niger* JaL303	*Talaromyces emersonii*	Yes
Glucoamylase	3.2.1.3	*Aspergillus niger* C878	*Trametes cingulata*	Yes
Alpha-amylase	3.2.1.1	*Aspergillus niger* C878	*Rhizomucor pusillus*	Yes
Alpha-amylase	3.2.1.1	*Aspergillus niger* C2218	*Rhizomucor pusillus*	Yes
Glucoamylase	3.2.1.3	*Aspergillus niger* C2218	*Gloeophyllum trabeum*	Yes
Glucoamylase	3.2.1.3	Aspergillus niger C2218	Penicillum oxalicum	Yes
Triacylglycerol lipase	3.1.1.3	Aspergillus niger C2218	*Candida antarctica*	Yes
Phospholipase A	3.1.1.32	Aspergillus niger C2948	*Talaromyces leycettanus*	Yes

Table 3 Novozymes products derived from *A. niger* strains where safety studies have been carried out.
[a)] The predecessor strain shows strains in the GM construction that are in common with the C40 strain lineage.
[b)] At least the following: *in vitro* test for gene mutations in bacteria (Ames); *in vitro* test for chromosomal aberration or *in vitro* micronucleus assay; 13 week sub chronic oral toxicity study in rats. The conclusions of these studies were in all cases favorable.

Novozymes' has evaluated this genetically modified Aspergillus niger production organism using the concepts initially outlined by Pariza and Foster, 1983 (2) and further developed by IFBC in 1990 (19), 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) and Pariza and Johnson in 2001. Novozymes uses the procedures outlined in the decision tree (Appendix 1) by Pariza and Johnson as a basis for our safety assessment.

The components of this evaluation: the identity of the recipient strain, a description of the incorporated DNA, the sources and functions of the introduced genetic material, an outline of the genetic construction of the production strain, and some characteristics of the production strain and the enzyme derived from it are given in Part 2.

The production strain is genetically modified by rDNA techniques as discussed in Part 2. The expressed enzyme product is a trehalase. The enzyme preparation is free of DNA encoding transferable antibiotic resistance 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 at two specific sites in the chromosome and the incorporated DNA is known not to encode or express any harmful or toxic substances.

Based on the information presented above, Novozymes has concluded that this production strain is considered to be derived from a safe lineage and is safe for use in the production of enzyme preparations for use in food.

6(b) Safety of the Donor Organism

The donor organism of the trehalase is Myceliophthora sepedonium. As indicated in Part 2, the introduced DNA is well defined and characterized. Only well characterized DNA fragments, limited solely to the trehalase 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 Trehalase Enzyme

As indicated in Part 2, the subject of this GRAS notification is a trehalase, EC 3.2.1.28.

Trehalase activity was first observed in Aspergillus niger in 1893 by Bourquelot (26) and has been isolated from dozens of other organisms. Trehalase is necessary for the catabolism of the trehalose molecule (27) (28). Trehalase enzyme can be found in many modern food sources that contain quantities of trehalose such as; honey, brewer's and baker's yeast, commercially grown mushrooms, oysters, fish and higher plants (26) (29). Trehalase is also found in humans as an intrinsic glycoprotein of the small intestine and renal brush-border membrane of enterocytes (30). Humans develop gut-associated trehalase in the early stages of life after conception. Concentrations of intestinal trehalase activity, in humans, are at a three to four-fold excess at birth. This suggests that trehalase is important in the early life of humans (29).

A review of the published literature shows that trehalase activity in humans has been studied over a period of several decades (31) (32) (33) (34) (35) (36) (37). The consensus of this literature is that trehalase is essential to the breakdown of trehalose, is found in a variety of foods commonly consumed by humans and is a natural constituent of the human gut.

A wide variety of enzymes are used in food processing (2) (3). And, enzyme proteins do not generally raise safety concerns (3) (2). Pariza and Foster (2) note that very few toxic agents have enzymatic properties. The safety of the trehalase was assessed using the Pariza and Johnson, (2001) decision tree which is included in this submission.

Based on the literature cited above and a safety evaluation of the trehalase enzyme preparation conducted by Novozymes, we conclude that the trehalase enzyme is safe for use in the distillation of ethanol.

6(d) Allergenic/Toxigenic Potential of the Trehalase 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.

1) 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.

2) 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) (38).

In order to further evaluate the possibility that the trehalase will cross-react with known allergens and induce a reaction in an already sensitized individual, a sequence homology to known food allergens was assessed. Following the guidelines developed by FAO/WHO, 2001 (39) and modified by Codex Alimentarius Commission, 2009 (40) the trehalase 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.

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 suitable gap penalty showed no matches. Alignment of the trehalase to each of the allergens and identity of hits with more than 35% identity over the full length of the alignment was performed using the global alignment "needle" in the program package EMBOSS (41) (42). No homology was found between the trehalase and any of the allergens from the databases mentioned above. And, a search for 100% identity over 8 contiguous amino acids was completed. Again, no homology was found.

The trehalase produced by *Aspergillus niger* sequence from C3085-1850-19 was assessed for its homology to known toxins. This assessment was based on the information present in the UNIPROT database (11-Feb-2016). This database

contains entries from SWISSPROT and TREMBL. The homology among the emerging entries was 14.8% indicating that the homology to any toxin sequence in this database is random and very low.

Consequently, oral intake of the trehalase is not anticipated to pose any food allergenic or toxigenic concerns.

6(e) Safety of the Manufacturing Process

This section describes the manufacturing process for the trehalase which follows standard industry practices (11) (10) (9). The quality management system used in the manufacturing process for the trehalase 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 use of the trehalase.

The following studies were performed on test batch PPT40595 with favourable results:

- Reverse Mutation Assay (Ames test)
- *In vitro* Cytotoxicity Test: Neutral Red Uptake
- 14-day oral (gavage) toxicity study

These tests are described in Appendix 2. Based on the presented toxicity data and the history of safe use for the *A. niger* strain it can be concluded that trehalase, represented by batch PPT40595, exhibits no toxicological effects under the experimental conditions described.

6(g) Results and Conclusion

Novozymes has reviewed the available data and information. We are not aware of any data and/or information that is, or appears to be, inconsistent with our conclusion of GRAS. Based on this critical review and evaluation, a history of safe use of *Aspergillus niger* and the limited and well defined nature of the genetic modifications, Novozymes concludes through scientific procedures that the subject of this notification; trehalase enzyme preparation, meets the appropriate food grade specifications and is produced in accordance with current good manufacturing

practices. Thus, it is generally recognized, among qualified experts, to be safe under the conditions of its intended use.

Part 7 – SUPPORTING DATA AND INFORMATION

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

APPENDICES

1. Pariza and Johnson Decision Tree Analysis

2. Summary of Toxicity Data, Trehalase PPT40595. 03 February 2017, File No. 2017-01916-01.

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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: a trehalase enzyme from Myceliophthora sepedonium produced in *Aspergillus niger*.

§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 trehalase enzyme will be used in ethanol distilling process. 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.

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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)

4/3/17

Janet Oesterling
Regulatory Affairs Specialist III

Date

Appendix 3- This trehalase from *Myceliophthora sepedonium* produced by a genetically modified strain of *Aspergillus niger* 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

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Department of Toxicology

Date : 03 February 2017
Ref.: JLIC
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SUMMARY OF TOXICITY DATA

Trehalase, batch PPT40595

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CONTENTS

1. ABSTRACT

The below series of toxicological tests were undertaken to evaluate the initial toxicological profile of Trehalase, batch PPT40595.

All studies were carried out in accordance with current OECD guidelines and in compliance with the OECD principles of Good Laboratory Practice (GLP) except for the in vitro cytotoxicity test. The studies were carried out at JAI RESEARCH FOUNDATION, India and Covance, UK during the period March 2016 to June 2016.

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

- Trehalase, batch PPT40595 was considered non-cytotoxic *in vitro* in a Neutral Red Uptake assay applying the mouse fibroblast cell line BALB/c cell culture.
- Trehalase, batch PPT40595 showed no mutagenic activity in the Ames test, either in the absence or presence of S9 indicating no genotoxicological potential.
- Trehalase, batch PPT40595 was considered well-tolerated when administered orally by gavage to rats for 14 days. The NOAEL (No Observed Adverse Effect Level) of both male and female rats was 8049.7 TNU(A)/kg body weight/day corresponding to 1297.7 mg TOS/kg body weight/day.

Based on the present toxicity data it can be concluded that Trehalase, batch PPT40595 exhibits no toxicological effects under the experimental conditions described.

2. TEST SUBSTANCE

Trehalase is a liquid enzyme concentrate containing a Trehalase (E.C. number 3.2.1.28). The non-reducing disaccharide trehalose is one of the most important storage carbohydrates, which is present in almost all forms of life except mammals. The disaccharide trehalose can be hydrolyzed into two molecules of glucose by the enzyme trehalase. The production organism is a genetically modified strain of Aspergillus niger. Aspergillus niger is ubiquitous in the environment and in general considered as a non-pathogenic fungus. This species has been widely used in industry and is known for its lack of potential to produce toxins.

2.1 Characterization

Trehalase, batch PPT40595 was used for the conduct of all the toxicological studies. The characterization of the toxbatch is presented in Table 1.

Table 1. Characterization data of Trehalase, batch PPT40595

Batch number	PPT40595
Activity	763 TNU(A)/g
Water (KF) (% w/w)	86.6
Dry matter (% w/w)	13.4
Ash (% w/w)	1.1
Total Organic Solids (TOS[1]) (% w/w)	12.3
Specific gravity (g/mL)	1.055

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

3. CYTOTOXICITY

3.1 *In Vitro* Cytotoxicity Test: Neutral Red Uptake in mouse fibroblast cell line BALB/c 3T3 culture[1]

The purpose of this study was to screen for cytotoxic potential of Trehalase, batch PPT40595 compared to the reference Amyloglucosidase, batch PPY35872.

The mouse fibroblast cell line BALB/c 3T3 was used as test system. After 48 hours incubation the test material was removed, wells washed and Neutral Red added to each well. Following incubation in a CO2 incubator and washing the aborbance at 540 nm (OD540) was measured to indicate the number of cells surviving exposure to the test material. The following concentrations were selected for Trehalse, batch PPT40595, for an accurate determination of cytotoxicity in this test model: 30-10- 3-1 and 0.3 mg test substance per ml growth medium.

SDS (Sodium Dodecyl Sulfate) was used as a positive control and the following concentrations were selected: 0.16, 0.13 and 0.10 mg/ml growth medium.

The concentration of the test substance required to reduce the viability of the treated test system to 50% of that of the untreated control test system was determined as the endpoint (NRU_{50}). The NRU_{50} value for the Trehalase, batch PPT40595, was estimated to be > 30 mg/ml.
The positive control, SDS, met the acceptance criteria of a valid test.

On the basis of the results of the present study, Trehalase batch PPT40595, was considered non-cytotoxic in the present *in vitro* Neutral Red Uptake assay.

4. MUTAGENICITY

4.1 Bacterial Reverse Mutation assay (Ames test)[2]

Trehalase, batch PPT40595 was examined for mutagenic activity in the bacterial reverse mutation assay using four histidine-requiring strains (TA98, TA100, TA1535 and TA1537) of Salmonella typhimurium, and one tryptophan requiring strain (WP2 uvrA pKM101) of Escherichia coli. Two separate experiments was performed and 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 batch of Trehalase, 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 Trehalase in liquid culture known as "treat and plate assay".

Experiment 1 treatments of all the tester strains were performed in the absence and in the presence of metabolic activation (S-9), using final concentrations of Trehalase batch PPT40595 at 16, 50, 160, 500, 1600 and 5000 µg TOS/mL, plus vehicle and positive controls. Following these treatments, no clear evidence of toxicity was observed, as would normally be manifest as a thinning of the background bacterial lawn and/or a marked reduction in revertant numbers.
Experiment 2 treatments of all the tester strains were performed in the absence and in the presence of S-9. The maximum test concentration of 5000 µg TOS/mL was retained for all strains. Narrowed concentration intervals were employed covering the range 150-5000 µg TOS/mL, in order to examine more closely those concentrations of Trehalase batch PPT40595 approaching the maximum test concentration and therefore considered most likely to provide evidence of any mutagenic activity. Following these treatments, no clear evidence of toxicity was observed.

The test article was completely soluble in the aqueous assay system at all concentrations treated, in each of the experiments performed.

Vehicle and positive control treatments were included for all strains in both experiments.

The mean numbers of revertant colonies all fell within acceptable ranges for vehicle control treatments, and were elevated by positive control treatments. No indication of any test article related amino acid feeding effects were observed on any of the test plates.

Following Trehalase batch PPT40595 treatments of all the test strains in the absence and presence of S-9, no clear and concentration-related increases in revertant numbers were observed that were ≥2-fold (in strains TA98, TA100 and WP2 uvrA pKM101) or ≥3 fold (in strains TA1535 and TA1537) the concurrent vehicle control. This study was therefore considered to have provided no evidence of any Trehalase batch PPT40595 mutagenic activity in this assay system.

It was concluded that Trehalase batch PPT40595 did not induce mutation in four histidine-requiring strains of Salmonella typhimurium, and one tryptophan requiring strain of Escherichia coli when tested under the conditions of this study.

5. IN VIVO TOXICOLOGY

5.1 – 14-day oral toxicity study in rats[3]

The study was conducted to determine the adverse effects of Trehalase, batch PPT40595, in Wistar rats, when administered orally through gavage for a period of 14 consecutive days. The methods followed were based on study outline provided by the Study Sponsor and inspired from the guideline of the OECD N° 407 (October 3, 2008).
A total of 20 male and 20 female Wistar rats were randomly divided into four groups, each group comprising 5 male and 5 female rats.
Rats were treated with Trehalase, batch PPT40595, for a period of 14 consecutive days through oral gavage at the dose levels of 805.0 TNU(A)/kg body weight/day (low dose), 2656.4 TNU(A)/kg body weight/day

(mid dose) and 8049.7 TNU(A)/kg body weight/day (high dose) corresponding to 129.8 mg TOS/kg body weight/day (low dose), 428.2 mg TOS/kg body weight/day (mid dose) and 1297.7 mg TOS/kg body weight/day (high dose), respectively.
Concurrent vehicle control group rats received reverse osmosis (RO) water alone. A fixed dose volume of 10 mL/kg/day was used. The dose formulation for mid- and low dose groups were prepared by dissolving the test item in RO water, and for the high dose group test item was used as such without dilution.

No mortality or signs of morbidity was observed during the study period. No visible clinical signs were observed throughout the study period. No significant changes were observed in mean body weight, mean body weight change and feed consumption of male and female rats treated with Trehalase, batch PPT40595 at low-, mid- and high dose levels when compared with the vehicle control group.
Minor statistically significant changes in MHC, WBC and absolute lymphocyte count were not considered to be treatment related findings due to absence of dose relationship and/or consistency between sexes.
The terminal body weights and organ weights (both absolute and relative) in animals belonging to all three treatment groups were well comparable with the concurrent vehicle control group.
External and internal examination of terminally sacrificed animals did not reveal any abnormalities.

In conclusion, Trehalase, Batch PPT40595 did not cause any adverse effects up to the highest dose level 8049.7 TNU(A)/kg body weight/day (corresponding to 1297.7 mg TOS/kg body weight/day when administered through oral gavage for 14 consecutive days in Wistar rats under the conditions and procedures followed in the present study. The NOAEL (No Observed Adverse Effect Level) for Trehalase, Batch PPT40595 of both male and female rats was 8049.7 TNU(A)/kg body weight/day corresponding to 1297.7 mg TOS/kg body weight/day.

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