A Maltogenic Alpha-Amylase from *Geobacillus stearothermophilus* Produced by *Bacillus licheniformis*

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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 maltogenic alpha-amylase produced by submerged fermentation of a genetically modified *Bacillus licheniformis* microorganism expressing the gene encoding for a maltogenic alpha-amylase from *Geobacillus stearothermophilus* (previously known as *Bacillus stearothermophilus*).

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

Systemic Name:	4-alpha-D-glucan alpha-maltohydrolase
Accepted Name:	glucan 1,4-alpha-maltohydrolase
EC No.:	3.2.1.133
CAS No.:	160611-47-2
Molecular Wt:	75 kDa
Specificity:	catalyzes the hydrolysis of 1,4-alpha-glylcosidic linkages in amylose, amylopectin, and related glucose polymers.
Amino acid sequence:	the total nucleotide and amino acid sequences have been determined

2.2 IDENTITY OF THE SOURCE

2.2(a) Production Strain

The *Bacillus licheniformis* production strain, designated HyGe767n2, was constructed via the intermediate strain MDT223. MDT223 is derived from a natural isolate of *Bacillus licheniformis* strain DSM 9552.

The expression plasmid used in the strain construction, contains strictly defined chromosomal DNA fragments and synthetic DNA linker sequences. The donor for the maltogenic alpha-amylase is *Geobacillus stearothermophilus.*

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).

2.2(b) Recipient Strain

The *Bacillus licheniformis* recipient strain, HyGe735, used in the construction of the maltogenic alpha-amylase production strain was modified at several chromosomal loci during strain development to inactivate genes encoding a number of proteases. Also, a

gene essential for sporulation was deleted, eliminating the ability to sporulate, along with the deletion of additional genes encoding unwanted proteins that can be present in the culture supernatant. The lack of these represents improvements in the product purity and stability.

2.2(c) Maltogenic Alpha-Amylase Expression Plasmid

The expression plasmid used to transform the *Bacillus licheniformis* recipient strain is based on the well-known *Bacillus* vectors pE194 (9) and pUB110 (10) from *Staphylococcus aureus*. No elements of these vectors are left in the production strain. The plasmid contains the expression cassette consisting of the *Bacillus amyloliquefaciens* and *Bacillus thuringiensis* engineered promoter, the *amyM* coding sequence and a transcriptional terminator.

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

2.2(d) Construction of the Recombinant Microorganism

In the construction of the production strain, *Bacillus licheniformis* HyGe767n2, the expression cassette was integrated into two loci by targeted homologous recombination. Targeted integration of the expression cassette at these loci allows the expression of the maltogenic alpha-amylase gene *amyM* from the promoter.

The resulting maltogenic alpha-amylase strain containing two copies of the *amyM* gene was named HyGe767n2.

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

2.2(e) Stability of the Introduced Genetic Sequences

The maltogenic alpha-amylase gene *amyM,* is stably integrated into the *Bacillus licheniformis* chromosome and as such, is poorly mobilized for genetic transfer to other organisms and is mitotically stable. The genetic stability of the production strain was confirmed by production of maltogenic alpha-amylase at industrial scale fermentation.

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 is satisfactorily addressed (4).

2.3 METHOD OF MANUFACTURE

This section describes the manufacturing process for the maltogenic alpha-amylase which follows standard industry practices (11) (12) (13). The quality management system used in the manufacturing process for the maltogenic alpha-amylase complies with the requirements of ISO 9001. It is produced under a standard manufacturing process as outlined by Aunstrup (12) and in accordance with current Good Manufacturing Practices, using ingredients that are accepted for general use in foods, and under conditions that ensure a controlled fermentation. The enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in the Food Chemicals Codex (14). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (15).

2.3(a) Raw Materials

The raw materials used in the fermentation and recovery process for the maltogenic alpha-amylase enzyme concentrate are standard ingredients used in the enzyme industry (13) (12) (11). The raw materials conform to Food Chemicals Codex specifications except those raw materials which do not appear in the FCC (16). For those not appearing in the FCC, internal specifications have been made in line with FCC requirements. On arrival at Novozymes A/S, the raw materials are sampled by the Quality Control Department and subjected to the appropriate analyses to ensure their conformance to specifications.

Any antifoams or flocculants used in fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 10, 1998. The maximum use level of the antifoams and/or flocculants, if used in the product, is not greater than 1%.

2.3(b) Fermentation Process

The maltogenic alpha-amylase is produced by pure culture submerged fed-batch fermentation of a genetically modified strain of *Bacillus licheniformis* as described in Part 2.

During fermentation, the maltogenic alpha-amylase enzyme produced by *Bacillus licheniformis* is secreted into the fermentation media.

All equipment is carefully designed, constructed, operated, cleaned, and maintained to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are done to ensure absence of foreign microorganisms and confirm strain identity.

2.3(c) Production Organism

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

2.3(d) Criteria for the Rejection of Fermentation Batches

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

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

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

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

Any contaminated fermentation is rejected.

2.3(e) Recovery Process

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

2.3(f) Purification Process

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

1) Pretreatment - pH adjustment and flocculation

2) Primary Separation – vacuum drum filtration or centrifugation

3) Concentration - ultrafiltration and/or evaporation

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

5) Preservation and Stabilization of the liquid enzyme concentrate

6) Final concentration – evaporation and/or ultrafiltration.

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

2.4 COMPOSITION AND SPECIFICATIONS

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

2.4(a) Quantitative Composition

Table 1 below identifies the substances that are considered diluents, stabilizers and inert raw materials used in the enzyme preparation. The fermentation media used in the manufacturing of the maltogenic alpha-amylase enzyme preparation does not contain any major food allergens.

Table 1. Typical composition raw materials of the enzyme preparations

Substance	Approximate Percentage
Wheat Flour	75 - 90%
Sodium Chloride	5 – 10%
Enzyme Solids (TOS*)	2.9%
Water	1 - 5%

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

2.4(b) Specifications

The maltogenic alpha-amylase enzyme preparation complies with the recommended purity specification criteria for “Enzyme Preparations” as described in *Food Chemicals Codex* (17). In addition, it also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications (15).

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

Table 2. Analytical data for three food enzyme batches.

Parameter	Specifications	PPY66188	PPY67385	PPY67546
Maltogenic Alpha-Amylase activity	MANU/g	19000	21600	17100
Total viable count	$\leq 10^4$/g	500	100	<100
Lead	<5 mg/kg	<5	<5	<5
Salmonella sp.	ND in 25g of sample	ND	ND	ND
Total coliforms	≤30/g	< 4	< 4	< 4
Escherichia coli	ND in 25 g of sample	ND	ND	ND
Antimicrobial activity	ND	ND	ND	ND
Production Organism	ND	ND	ND	ND

*ND: Not Detected

2.5 PHYSICAL OR TECHNICAL EFFECT

2.5(a) Mode of Action and Application

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

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

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

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

2.5(b) Use Levels

The maltogenic alpha-amylase is not added to the final foodstuffs. It is used as a processing aid during food manufacturing to hydrolyse starch.

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

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

Starch processing

Up to 16500 MANU per kilogram of starch dry matter

Baking processes and other cereal based processes

Up to 15000 MANU per kilogram of starch dry matter.

Brewing processes and other cereal based beverage processes

Up to 16000 MANU per kilogram of starch dry matter.

2.5(c) Enzyme Residues in the Final Food

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

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

For baked goods, the production process includes mixing wheat flour, or other cereal flour, with additional ingredients and water in preparation of a dough or batter. The maltogenic alpha-amylase is added along with the other raw materials. This dough/batter is allowed to rest or ferment and then is shaped into the appropriate formed food. The dough/batter is then either baked, boiled or steamed at high temperatures. The enzyme performs its function during the dough handling and in the first part of the baking process at which time the enzyme is denatured by the high temperatures during this process.

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

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

The enzyme used during all these processes does not exert any unintentional enzymatic activity in the final food. This is due to various factors such as; denaturation of the enzyme during processing, depletion of the substrate, lack of

water activity, wrong pH, etc. which are based on the specific application and process conditions used by the individual food producer. In some cases, such as distilling, the enzyme may no longer be present in the final food due to the harsh processing steps applied during manufacturing of the final food.

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

PART 3 - DIETARY EXPOSURE

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

3(a) Assumptions in Dietary Exposure

The assumptions are highly exaggerated since the enzyme protein and the other compositional substances are diluted or removed during certain processing steps. Furthermore, all processed foods and beverages produced with the enzyme are not always produced with the maximum recommended dosage. Overall, the human exposure to the maltogenic alpha-amylase will be negligible. The enzyme preparation is used as a processing aid and in very low dosages.

Therefore, the safety margin calculation derived from this method is highly exaggerated.

3(b) Food Consumption Data

The exposure assessment was performed using the Budget Method (18) (19) (20). The Budget Method assumptions represent a "maximum worst case" scenario of human consumption. The maltogenic alpha-amylase enzyme is assumed to be used at its maximum recommended dosages in the production of all processed beverages. It is also assumed that the totality of the maltogenic alpha-amylase enzyme will end up in the final food.

Assumptions in the Budget Method

The maltogenic alpha-amylase concentrate has an average activity of 19233 MANU/g and approximate 2.9% TOS (Total Organic Solids) content.

This corresponds to an activity/TOS ratio of 663 MANU/mg TOS.

The maximum recommended dosage used to calculate the dietary exposure is 16500 MANU per kg starch derived dry matter.

This corresponds to 25mg TOS

Solid Food: The maximum energy intake over the course of a lifetime is 50 kcal/kg body weight (b/w) /day. Fifty kcal corresponds to 25g food.

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

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

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

25mg TOS per kg/1000g per kg x 3.12g = 0.08mg TOS

Liquids: The maximum intake of liquids (other than milk) is 100 ml/kg body weight day. Assuming that 25% of the non-milk beverages are processed, the daily consumption will be 25 ml processed beverages per kg body weight.

It is further assumed that all processed beverages contain 13% starch (or starch-derived) dry matter = 3.25g starch derived dry matter per kg bw per day.

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

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

25mg TOS per kg/1000g per kg x 3.25g = 0.08mg TOS

Theoretical Maximum Daily Intake (TMDI)

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

0.08 + 0.08 = 0.16mg TOS/kg body weight/day

Margin of Safety

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

The NOAEL dose level in the 13-week oral toxicity study in rats conducted on alpha-amylase tox batch PPY34423 was the highest dosage possible, 796mg TOS/kg bw/day. See Table 3 below.

Table 3. Calculation of the Margin of Safety

NOAEL (mg TOS/kg bw/day)	796
*TMDI (mg TOS/kg bw/day)	0.16
Margin of Safety	4,975

*based on the worst-case scenario

PART 4 - SELF-LIMITING LEVELS OF USE

This part does not apply

PART 5 - COMMON USE IN FOOD BEFORE 1958

This part does not apply

PART 6 - NARRATIVE ON THE CONCLUSION OF GRAS STATUS

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

6(a) Safety of the Manufacturing Process

This Part describes the manufacturing process for the maltogenic alpha-amylase, which follows standard industry practices (13) (11) (12).

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

The enzyme preparation complies with the purity criteria recommended for enzyme preparations as described in the Food Chemicals Codex (14). It also conforms to the General Specifications for Enzyme Preparations Used in Food as proposed by JECFA (15).

6(b) 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).

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, are safe to consume (4). 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” (2).

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

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

The enzyme preparation is free of DNA encoding transferable antibiotic resistance gene DNA. The introduced DNA is well characterized and safe for the construction of microorganisms to be used in the production of food grade products. The DNA is stably integrated into the chromosome at specific sites in the chromosome and the incorporated DNA is known not to encode or express any harmful or toxic substances.

Bacillus licheniformis has a long history of safe industrial use in the production of enzymes used in human food. It is widely recognized as a harmless contaminant found in many foods (4). *Bacillus licheniformis* is not a human pathogen and it is not toxigenic (21).

Bacillus licheniformis has been used in the fermentation industry for the production of enzymes, antibiotics, and other specialty chemicals. Various enzymes are produced by *Bacillus licheniformis* and are considered GRAS substances (GRNs 265, 277, 472, 572, 587, 645) (22).

In addition, it has also been granted a Qualified Presumption of Safety status by the European Food Safety Authority (23).

6(c) Safe Strain Lineage

The safety of this *Bacillus licheniformis* production strain was established following published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients (3) (4). The *Bacillus licheniformis* production strain is derived from a safe strain lineage that is comprised of production strains for enzyme preparations which have full toxicological safety studies (i.e. 13-week oral toxicity study in rats, Ames test and chromosomal aberration test or micronucleus assay).

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

Table 4: Safe Strain Lineage

Enzyme	EC No.	Predecessor strain[1]	Donor strain	Safety studies[2]
Alpha-amylase (GRASP 0G0363)	3.2.1.1	*Bacillus licheniformis* Si3	*Bacillus stearothermophilus*	Yes
Alpha-amylase (GRN 22)	3.2.1.1	*Bacillus licheniformis* SJ1707	*Bacillus licheniformis*	Yes
Cyclodextrin glucanotransferase	2.4.1.19	*Bacillus licheniformis* SJ1707	*Thermoanaerobacter sp.*	Yes

Alpha-amylase	3.2.1.1	*Bacillus licheniformis* SJ1707	*Bacillus licheniformis*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* SJ1904	*Bacillus licheniformis*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* MDT223	*Bacillus stearothermophilus*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* MDT223	*Bacillus amyloliquefaciens*	Yes
Serine protease (GRN 564)	3.4.21.1	*Bacillus licheniformis* MDT223	*Nocardiopsis prasina*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis* MDT223	*Bacillus licheniformis*	Yes
Xylanase (GRN 472)	3.2.1.8	*Bacillus licheniformis* MDT223	*Bacillus licheniformis*	Yes
L-Glutaminase (GRN774)	3.5.1.2	*Bacillus licheniformis MDT223*	*Bacillus licheniformis*	Yes
Alpha-amylase	3.2.1.1	*Bacillus licheniformis PP3579*	*Bacillus licheniformis*	Yes
Beta-amylase	3.2.1.1	*Bacillus licheniformis* PP3579	*Bacillus flexus*	Yes
Beta-galactosidase (GRN 572)	3.2.1.23	*Bacillus licheniformis* AEB1763	*Bifidobacterium bifidum*	Yes
Acetolactate decarboxylase (GRN 587)	4.1.1.5	*Bacillus licheniformis* AEB1763	*Bacillus brevis*	Yes
Pullulanase (GRN 645)	3.2.1.41	*Bacillus licheniformis* AEB1763	*Bacillus deramificans*	Yes
PI-phospholipase C (GRN 728)	3.1.4.11	*Bacillus licheniformis* AEB1763	*Pseudomonas sp-62186*	Yes
Phospholipase-C (GRN 689)	3.1.4.3	*Bacillus licheniformis AEB1763*	*Bacillus thuringiensis*	Yes

Table 4. Novozymes products derived from *B. licheniformis* strains. [1]The predecessor strains show common strains in the GM construction pathway. [2]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.

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

The fact that no issues are observed in safety studies on different enzymes (e.g. amylases, protease, xylanase) produced by strains derived from a common predecessor (B. licheniformis MDT223), strongly supports the safety of the B. licheniformis strain lineage, independent of which enzyme is produced.

Similarly, no safety issues are observed in safety studies performed on the same type of enzyme (amylase) derived from different predecessor strains (Si3, SJ1707, SJ1904, MDT223, PP3579). This emphasizes that the GM construction steps performed on the strains do not cause safety issues.

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

used to modify the host organism are well defined and commonly used. Therefore, the elements needed to establish a safe strain lineage as defined in Pariza and Johnson, 2001 (3) have been met.

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

6(d) Safety of the Donor Organism

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

The donor organism of the maltogenic alpha-amylase is *Geobacillus stearothermophilus*. As indicated in Part 2 the introduced DNA is well defined and characterized. The introduced DNA does not code for any known harmful or toxic substances.

6(e) Safety of the Maltogenic Alpha Amylase Enzyme

The principal enzyme activity is maltogenic alpha amylase, a subgroup of alpha-amylases.

The maltogenic alpha amylase, object of this notification, belongs to the group alpha-amylases. Maltogenic alpha amylase, catalyze the hydrolysis of 1,4-alpha-glycosidic linkages in starch polysaccharides, amylose and amylopectin and hydrolysis-products of starch of various chain length such as dextrins and maltodextrins. The main reaction product in the hydrolysis is maltose.

A wide variety of enzymes, including maltogenic alpha amylases, are used in food processing with a majority of use in the hydrolysis of starch (3) (2). Maltogenic alpha amylases have been used in food applications, particularly baking, since the 1900s (24) (25). Maltogenic alpha-amylases are also used in the dairy industry as a catalyst for starch to maltose conversion (24) (26). They are universally distributed throughout the animal, plant and microbial kingdom (bacteria and fungi) and are naturally present in many raw materials including wheat, barley and malt (27).

Also, there are several maltogenic alpha-amylase enzyme preparations; GRNs 842, 751 and 746, all having been concluded as GRAS by the FDA, and were responded to with “no questions” (22). The maltogenic alpha-amylase gene (*amyM*), described in Part 2 of this notification, is the same gene used in the strain construction described in GRN 751.

All of these notifications have provided sufficient toxicological testing data showing evidence that there is no toxicological concern regarding the safety and consumption of maltogenic alpha-amylase enzymes.

A literature search was performed in September 2020 for the period of 2017 to 2020 on maltogenic amylase utilizing the database *Web of Science* and key words "maltogenic alpha amylase", "food safety", "toxicity", "human consumption" and "safety". A total of 36 relevant hits were found. Novozymes reviewed the totality of the available abstracts and found none to be inconsistent with our conclusion of the general recognition of safety of maltogenic alpha amylase.

Because of the long history of use of maltogenic alpha-amylase in food it is our conclusion that the maltogenic alpha-amylase, subject of this notification, is safe for use as a processing aid in food.

6(f) Allergenic/Toxigenic Potential of the Maltogenic Alpha-Amylase Enzyme

The ingestion of a food enzyme protein is not considered a food allergy concern. This is based on the following considerations:

1) Enzymes have a long history of safe use in food, with no indication of adverse effects or reactions.

2) The majority of proteins are not food allergens. A wide variety of enzyme classes and structures are naturally present in plant and animal based foods, and based on previous experience, food enzymes are not homologues to known allergens, which make it very unlikely that a new enzyme would be a food allergen.

3) Enzymes in foods are added in concentrations in the low range of part per millions. The enzyme is typically removed or denatured during food processing and denatured protein has been shown to be very susceptible to digestion in the gastro-intestinal system. Moreover, a wide range of naturally occurring food enzymes have been shown to be very labile in the gastro-intestinal system even in the native unprocessed form.

The above statements are further supported by the publication: "Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry" (Bindslev-Jensen *et al*, 2006) (28).

In order to further evaluate the possibility that the maltogenic alpha-amylase will cross-react with known allergens and induce a reaction in an already sensitized individual, a sequence homology to known allergens was assessed. Following the guidelines developed by FAO/WHO, 2001 (29) and modified by Codex Alimentarius Commission, 2009 (17) the maltogenic alpha-amylase was compared to allergens from the FARRP allergen protein database (http://allergenonline.org) as well as the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee (**http://www.allergen.org**).

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

The analyses identified one mosquito (Aed a 4) and three fungal (Asp o 21, Asp f 13 and Sch c 1) allergens, having an identity with maltogenic alpha-amylase above the threshold of 35% across an 80-amino acid window.

None of the hits, Asp o 21, Asp f 13, Sch c 1 and Aed a 4, are registered as food allergens (**http://www.allergen.org**). Further, an additional screen of the current literature did not find any evidence that Asp f 13, Sch c 1 or Aed a 4 can trigger oral sensitization. The Asp o 21 alpha-amylase very rarely causes oral sensitization and only few cases of potential food allergy to the ingested Asp o 21 alpha-amylase have been described whereof, three were linked to occupational sensitization. (30) (31) (32) (33).

In addition, the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) Working Group on Consumer Allergy Risk from Enzyme Residues in Food, performed an in-depth analysis of the allergenicity of enzyme products. In this paper, Dauvrin and colleagues conclude that enzyme exposure by ingestion, in opposition to exposure by inhalation, is extremely unlikely to lead to sensitization. There is compelling evidence that persons affected by occupational asthma can ingest the respiratory allergen without acquiring clinical symptoms of food allergy, suggesting that inhalation is not likely to result in food allergy. Only one single case has been reported in the literature and this case was not verified as a bona fide oral sensitization to enzymes in food (34).

This is backed up by the study conducted by Bindslev-Jensen et al (28) using the generally recognized guidelines for food allergy diagnosis (skin prick test, specific serum IgE and DBPCFC). This study included 400 patients with a diagnosed allergy to one or more of inhalation allergens, food allergens, bee or wasp allergens. The study concluded that no cases of IgE-mediated food allergy to commercial enzymes (including alpha-amylases) could be found. Further, there were no indications of cross-reactivity between the tested enzymes used in food and the main known allergens causing clinical symptoms in the patients included in the study.

Also, a search for 100% identity over 8 contiguous amino acids was completed. No homology was found. And, a search for homology of the maltogenic alpha-amylase sequence to known toxins was assessed based on the information present in the UNIPROT database (2019-03-05). This database contains entries from SWISSPROT and TREMBL. The homology among the emerging entries was below 17%, indicating that the homology to any toxin sequence in this database is low and random.

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

6(g) Safety Studies Conducted

Novozymes has repeatedly used the procedures outlined by Pariza and Johnson to evaluate enzymes derived from *Bacillus licheniformis* production strains (3). As described in Part 6(c), Novozymes has concluded, that strains within the safe strain lineage of *Bacillus licheniformis* pose no safety concerns. Table 4 lists the strains within this lineage, with many having corresponding GRNs on file with the FDA, where toxicological safety studies have been performed.

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

It is reasonable to expect and conclude that enzymes produced by *Bacillus licheniformis* strains within this safe strain lineage will show similar toxicological profiles and further supports our conclusion that *Bacillus licheniformis* strains are safe hosts for the expression of enzymes. Therefore, we believe additional toxicological studies on the article of commerce (subject of this notification) are of little to no value, redundant and considered unnecessary (35) (36).

6(h) Description of the Test Article

The *Bacillus licheniformis* production strain (HyGe767n2) for the maltogenic alpha-amylase enzyme (subject of this notification), was developed from the safe strain lineage listed in Table 4.

Novozymes has determined that the results of the toxicology studies on alpha-amylase enzyme concentrate, batch PPY34423 from *Bacillus licheniformis*, can be bridged to support the toxicological outcome of the maltogenic alpha-amylase from *Bacillus licheniformis*, the subject of this notification.

This approach is in line with the Safe Strain Lineage concept as outlined by Pariza and Johnson, the EFSA (European Food Safety Authority) opinion (Question No. EFSA-Q-2013-00895) and the EFSA CEF Guidance on Food Enzymes regarding toxicological testing (37) (38) (39).

Based on the genetic modification performed and described in Part 2 of this dossier, the recipient strain, used to construct the production strain HyGe767n2, is closely related to the predecessor strain PP3579 mentioned in Table 4. There is no reason

to assume that the recombinant production strain should be less safe than the PP3579 strain.

All toxicology studies performed on the alpha-amylase enzyme concentrate, batch PPY34423, from the *Bacillus licheniformis* production strain, were carried out in accordance with current OECD guidelines and in compliance with the OECD principles of Good Laboratory Practice (GLP). The studies include a bacterial reverse mutation assay, *in vitro* micronucleus assay and a 13-week oral (gavage) study in rats. The studies were performed at Novozymes A/S (Denmark), Huntingdon Life Sciences (UK) and Covance Laboratories Ltd. (UK) during the period November 2013 to July 2014.

A summary of the toxicology studies for the alpha-amylase enzyme concentrate batch PPY34423, produced by *Bacillus licheniformis*, is included in Appendix 2.

Based on the presented toxicity data, the history of safe use and the safe strain lineage of the *Bacillus licheniformis* production strain, it can be concluded that the test preparation; enzyme concentrate batch PPY34423, exhibits no toxicological effects under the experimental conditions described in the summary

6(i) Results and Conclusion

The enzyme industry has performed hundreds of toxicology studies using a variety of enzymes (e.g. amylases, glucanases, lipases etc.) derived from multiple production organisms (e.g. *Bacillus licheniformis*, *Trichoderma reesei, Bacillus subtilis* etc.) with no adverse findings observed in the conducted studies (3) (40) (41).

Results of the toxicity and mutagenicity tests described in Appendix 2 showed no toxicity or mutagenicity of the test article, enzyme concentrate batch PPY34423 produced by *Bacillus licheniformis*.

A critical review and evaluation of the maltogenic alpha-amylase enzyme preparation (subject of this notification) and the alpha-amylase enzyme preparation (subject of the test material PPY34423) was done following the concepts of the Pariza papers and the recently described process for the evaluation of GRAS for industrial microbial enzymes by Sewalt et al. (42) (2) (3).

Based on the published, publicly available scientific information about *Bacillus licheniformis* production strains and maltogenic alpha-amylase enzymes used in food processing, along with the supporting data generated by Novozymes and using the decision tree evaluation method outlined by Pariza and Johnson (39), Novozymes considers the maltogenic alpha-amylase enzyme preparation (subject of this notification), produced by the *Bacillus licheniformis* production organism to be generally recognized as safe.

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, *Amylase* from *Bacillus licheniformis*, batch PPY34423. January 15, 2014, File No. 2014-00726-01.

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

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From: Srinivasan, Jannavi
To: JAO (Janet Oesterling)
Subject: RE: [EXTERNAL] GRN 975 Review
Date: Thursday, May 20, 2021 9:25:00 AM

Thank you, Janet. You have understood the question correctly. The FCC edition is accurate; I apologize for flagging that inadvertently.
Thank you!
Jannavi

From: JAO (Janet Oesterling) <JAO@novozymes.com>
Sent: Wednesday, May 19, 2021 2:19 PM
To: Srinivasan, Jannavi <Jannavi.Srinivasan@fda.hhs.gov>
Subject: [EXTERNAL] GRN 975 Review

Hello Jannavi,

I am doing well, thank you for asking. Hope you are also well and looking forward to summertime. Below, in red, is my response to your inquiry.

1. Please confirm that the promoter consisted of an engineered promoter containing sequences from both *B. amyloliqufaciens* and *B. thuringiensis*. Please also identify the source of the transcriptional terminator. It is confirmed that the promoter consists of an engineered promoter containing sequences from both *B. amyloliqufaciens* and *B. thuringiensis.* The identity of the source of the transcriptional terminator is *B. clausii and B. licheniformis.*
2. Please update the edition of the Food Chemical Codex. The edition currently listed in the References section, 12th, is to my knowledge the edition currently released. The next edition, 13th, will not be published until March 2022. Perhaps I have misunderstood your request?

FCC Publication and Comment Schedule

FCC Forum is the process for new and revised *Food Chemicals Codex* public review and comment. All proposed standards and revisions for the FCC are first posted in the free, online FCC Forum for a 90-day public comment period. Once revisions or new standards are approved by the Food Ingredients Expert Committee they are published in the FCC main edition or its Supplements. The table below shows applicable FCC Forum comment periods, targeted publications, and publication dates.

FCC Forum Date	Comment Period	Target FCC Publication	Publication Date	Publication Effective Date
Dec 2019	Dec 31 2019-Mar 31, 2020	FCC, Twelfth Edition, First Supplement	Sep 1, 2020	Dec 1, 2020
Jun 2020	Jun 30-Sep 30, 2020	FCC, Twelfth Edition, Second Supplement	Mar 1, 2021	Jun 1, 2021
Dec 2020	Dec 31, 2020- Mar 31, 2021	FCC, Twelfth Edition, Third Supplement	Sep 1, 2021	Dec 1, 2021
Jun 2021	Jun 30- Sep 30, 2021	FCC, Thirteenth Edition	Mar 1, 2022	Jun 1, 2022
Dec 2021	Dec 21, 2021-Mar 31, 2022	FCC, Thirteenth Edition, First Supplement	Sep 1, 2022	Dec 1, 2022
Jun 2022	June 30- Sep 30, 2022	FCC, Thirteenth Edition, Second Supplement	Mar 1, 2023	Jun 1, 2023
Dec 2022	Dec 31, 2022- Mar 31, 2023	FCC, Thirteenth Edition, Third Supplement	Sep 1, 2023	Dec 1, 2023

Information in the above table is subject to change as needed and without prior notice.

Best regards,

Janet Oesterling
Regulatory Affairs Specialist III

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From: Srinivasan, Jannavi <Jannavi.Srinivasan@fda.hhs.gov>
Sent: Friday, May 14, 2021 7:02 PM
To: JAO (Janet Oesterling) <JAO@novozymes.com>
Subject: GRN 975 Review

Dear Janet,

I hope this finds you safe and well.

Please find below some questions that have come up during our review of GRN 975.

1. Please confirm that the promoter consisted of an engineered promoter containing sequences from both *B. amyloliqufaciens* and *B. thuringiensis*. Please also identify the source of the transcriptional terminator.
2. Please update the edition of the Food Chemical Codex.

Please let me know if you can respond within 10 business days.

Thank you!

Jannavi

Jannavi R. Srinivasan, Ph.D.
(she/her)
Office of Food Additive Safety
5001 Campus Dr.
College Park MD-20740
Ph: 2404021199

The secret is to work less as individuals and more as a team - Knute Rockne