ORIGINAL SUBMISSION

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#464

GRN 000664

July 15, 2016

Dr. Paulette Gaynor
Office of Food Additive Safety (HFS-255)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

RE: GRAS Notification - Exemption Claim

Dear Dr. Gaynor,

Pursuant to the proposed 21C.F.R. §170.36 (c) (l) Danisco US Inc. (operating as DuPont Industrial Biosciences) hereby claims that α-amylase enzyme preparation from *Bacillus licheniformis* is Generally Recognized as Safe; therefore, it is exempt from statutory premarket approval requirements.

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

Proposed § 170.36 (c)(l)(i) The name and address of the notifier

Danisco US Inc.
(Operating as DuPont Industrial Biosciences)
925 Page Mill Road
Palo Alto, CA 94304

Proposed § 170.36 (c)(l)(ii) The common or usual name of notified substance

Alpha-amylase enzyme preparation from *Bacillus licheniformis*

Proposed § 170.36 (c)(l)(iii) Applicable conditions of use

The α-amylase is GRAS for use as a processing aid in carbohydrate processing, to produce sugar syrups and in fermentation to produce products such as potable alcohol and organic acids (i.e. citric and lactic).

Proposed §170.36 (c)(l)(iv) Basis for GRAS determination

This GRAS determination is based upon scientific procedures.

Proposed § 170.36 (c)(l)(v) Availability of information

A notification package providing a summary of the information that supports this GRAS determination is enclosed with this notice. The package includes a safety evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure. The complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying upon request.

If you have questions or require additional information, please contact me at 650-846-5861 or fax at 650-845-6502.

Sincerely, (b) (6)

(b) (6)

Vincent Sewalt, PhD
Senior Director, Product Stewardship & Regulatory
Danisco US Inc.
(operating as DuPont Industrial Biosciences)
650-846-5861 / vincent.sewalt@dupont.com

Enclosures (3 binders)

An Alpha-amylase Enzyme

Preparation Derived from

Bacillus licheniformis

Expressing the Alpha-amylase Gene

From

Cytophaga sp.

Is Generally Recognized As Safe

For Use in Food Processing

Notification Submitted by Danisco US Inc.
(operating as DuPont Industrial Biosciences)

July 15, 2016

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OFFICE OF
FOOD ADDITIVE SAFETY

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1. GENERAL INTRODUCTION

The α-amylase preparation under consideration is derived from a non-pathogenic, non-toxigenic strain of *Bacillus licheniformis* (strain JML 1584), which has been genetically modified to express a variant α-amylase gene from *Cytophaga* sp. Descriptions of the genetic modification, production methods, risk assessment, and characterization of the enzyme product follow. The α-amylase enzyme is herein designated as C16F α-amylase.

The enzyme is intended for use in carbohydrate processing, including starch hydrolysis to manufacture sweeteners such as high fructose corn syrup (HFCS) and specialty starches, and to produce fermentable carbohydrate for yeast fermentation to produce organic acids (i.e. lactic and citric acid) and potable alcohol.

In these applications, the *Cytophaga* sp. α-amylase will primarily be replacing α-amylase from one of the other commercial sources. In all of these applications, the α-amylase will be used as a processing aid where the enzyme is either not present in the final food or present as inactive protein in insignificant quantities having no function or technical effect in the final food. Pursuant to 21CFR170.30 (i) (Appendix 1) that establishes a manufacturer's responsibility to independently establish that a use of a product not stated in an existing GRAS affirmation is GRAS, DuPont independently evaluated the safety of the C16F α-amylase for such uses.

Given the world-wide use of enzymes in food processing and recent scientific advances, primarily in the fields of molecular biology and protein engineering, guidelines for current and future food safety evaluations of enzyme preparations for use in human and animal food have been published (Pariza and Foster, 1983; Pariza and Johnson, 2001). These guidelines provide a peer-reviewed decision tree process for the determination of the safety of enzyme preparations used in food.

To assess the safety of the C16F α-amylase for use in the applications listed above, DuPont vigorously applied the criteria identified in the guidelines, utilizing enzyme toxicology/safety data, the history of safe use of enzyme preparations from *B. licheniformis* and of other α-amylases in food, the history of safe use of the production organism for the production of enzymes used in food, and a comprehensive survey of the scientific literature. Based on these sources pursuant to FDA proposed regulation, proposed 21CFR170.36 (Appendix 1), DuPont has determined, based on scientific procedures including analysis of publicly available information, that the C16F α-amylase preparation derived from *B. licheniformis,* strain JML1584, is safe and suitable for use in carbohydrate processing, including starch hydrolysis to manufacture sweeteners (such as HFCS), specialty starches, and to produce fermentable carbohydrate for yeast fermentation to produce organic acids (i.e. lactic and citric acid) and potable alcohol.

1.1 Exemption from Pre-market Approval

Pursuant to the regulatory and scientific procedures established in proposed 21 C.F.R. 170.36 (Appendix 1), DuPont Industrial Biosciences has determined that its α-amylase enzyme preparation produced by *Bacillus licheniformis* expressing the gene encoding α-amylase from *Cytophaga* sp. is a Generally Recognized as Safe ("GRAS") substance for the intended food application and is, therefore, exempt from the requirement for premarket approval.

1.2 Name and Address of Notifier

Danisco US Inc.
(Operating as DuPont Industrial Biosciences)
925 Page Mill Road
Palo Alto, CA 94304

1.3 Common or Usual Name of Substance

The α-amylase enzyme preparation is from *Bacillus licheniformis* expressing the gene encoding the α-amylase from *Cytophaga* sp. (C16F α-amylase).

1.4 Applicable Conditions of Use

The α-amylase is GRAS for use as a processing aid in carbohydrate processing, to produce sugar syrups and in fermentation to produce products such as potable alcohol and organic acids (i.e. citric and lactic).

1.5 Basis for GRAS Determination

This GRAS determination is based upon scientific procedures.

1.6 Availability of Information for FDA Review

A notification package providing a summary of the information that supports this GRAS determination is enclosed with this notice. The package includes a safety evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure. The complete data and information that are the basis for this GRAS determination are available for review and copying at 925 Page Mill Road, Palo Alto, CA 94304 or will be sent to the Food and Drug Administration upon request.

2. PRODUCTION ORGANISM

2.1 Production Strain

The production strain is derived from *Bacillus licheniformis* strain JML1584, which has been genetically modified to express an optimized variant α-amylase gene from *Cytophaga* sp. *Cytophaga sp.* is part of the *Cytophaga-Flavobacteria* cluster, which can be found globally in every habitat in the biosphere (Kirchman, 2002). Although, *Cytophaga* sp. is prevalent in the soil, it can also be found in coastal water, offshore water, sediments, hydrothermal vents and the polar region (Alonso *et. al.*, 2007). In these ecosystems, the group can be found free living, attached to organic compounds and associated with marine plankton and animals (Alonso *et. al.*, 2007). *B. licheniformis* is a well-characterized organism with a long history of use in industrial applications. An extensive environmental and human risk assessment of *B. licheniformis*, including its history of commercial use has been published by the US Environmental Protection Agency (1997). It was concluded that *B. licheniformis* is not a human pathogen nor is it toxigenic. Moreover, the production strain pertains to a safe strain lineage as defined by Pariza and Johnson (2001), see Appendix 5.

2.2 Host Microorganism

The original host strain is *B. licheniformis* Bra7, which was developed from its wild-type parent by classical strain improvement only, for optimal α-amylase production and lowered protease production. The strain *B. licheniformis* Bra7 and strains derived from it have been in use for industrial scale production of α-amylase for food processing applications since 1989, with food grade versions in use for grain processing since 1998. *Bacillus licheniformis* has been used for decades in the production of food enzymes with no known reports of adverse effects to human health or the environment (de Boer and Diderichsen, 1994). The US Food and Drug Administration reviewed the safe use of food-processing enzymes from well-characterized recombinant microorganisms, including *B. licheniformis* (Olempska-Beer *et al.* 2006). It was concluded that *B. licheniformis* is not a human pathogen nor is it toxigenic. It is also considered as suitable for Good Industrial Large Scale Practice (GILSP) worldwide and meets the criteria for a safe production microorganism as described by Pariza and Johnson (2001).

2.3 Donor Microorganism

The donor strain used as a source for the α-amylase sequence is a *Cytophaga* sp., a soil bacterium described by Jeang *et al.* (1995) and Jeang *et al.,* (2002). This *Cytophaga* sp. produces an α-amylase that shows the highest amino acid sequence similarity, 81 %, to α-amylase from *Bacillus* sp. 406. The gene inserted into the production organism was not isolated from the donor strain, but instead the gene encoding an optimized variant of this α-amylase was synthesized *in vitro* by GeneArt (Regensburg, Germany). As such, there are no concerns with regard to inadvertent transfer of DNA encoding for traits related to pathogenicity or toxicity. This specific variant of *Cytophaga* sp. α-amylase is referred to as C16F.

2.4 Alpha-amylase Expression Cassettes

The genetic modification of the *B. licheniformis* host involved recombinant DNA techniques to introduce a gene encoding an optimized variant of α-amylase (C16F) synthesized *in vitro* from *Cytophaga* sp., into the *B. licheniformis* Bra 7 host. Further genetic modifications were performed on the host strain by inactivation of the genes encoding α-amylase (*amyL*), chloramphenicol resistance (*cat*), a sporulation gene (*spo*IIAC), the subtilisin gene (*aprL*) and the glutamic acid specific protease gene (*mpr*). Next, the α-amylase encoding gene (*amy*) of *Cytophaga* sp. was synthesized with changes leading to seven amino acid modifications. The coding sequence of this gene was placed under the expression signals of the endogenous *B. licheniformis amyL* gene and the *B. subtilis aprE* 5'UTR, cloned in a vector derived from *Bacillus* plasmids pUB110 and pE194, together with the native *B. licheniformis cat* gene. The resulting plasmid was integrated into the host chromosome at the *cat* locus by Campbell type recombination. After integration, all vector sequences of the plasmid were deleted by recombination between direct repeated *cat* sequences. This cassette was amplified using several rounds of growth at increasing concentrations of chloramphenicol to obtain the final production strain. The final result is a strain in which only the *Cytophaga* sp. *amy* gene and the native *cat* gene were introduced into the host strain. The genetic construction was evaluated at every step to assess the incorporation of the desired functional genetic information and the intended chromosomal modifications were confirmed by PCR analyses.

2.5 Stability of the Introduced Genetic Sequences

The production strain is completely stable after industrial scale fermentation as judged by α-amylase production using the production organism containing the integrated expression cassettes.

2.6 Antibiotic Resistance Gene

No new antibiotic resistance genes were introduced in the construction of the production microorganism.

2.7 Absence of the Production Organism in the Product

The absence of the production microorganism is an established specification for the commercial product at a detection limit of <1 CFU/g. The production organism does not end up in food and therefore, the first step in the safety assessment as described by IFBC (1990) is satisfactorily addressed.

3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1 Enzyme Identity

IUB Nomenclature	Alpha-amylase
IUB Number:	3.2.1.1
CAS Number:	9000-90-2
EINECS Number:	232-565-6
Reaction catalyzed:	Endohydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides containing three or more (1→4)-α-linked D-glucose units.
Other names:	Glycogenase

3.2 Amino Acid Sequence

The variant *Cytophaga* sp. amino acid sequence of the C16F α-amylase differs from wild-type by only seven amino acids, and is included in Appendix 2. The amino acid modifications did not impact the integrity, functionality or safety of the protein. The sequence of the variant C16F α-amylase is similar to various other α-amylases isolated from commercially relevant bacteria, e.g., it is 81% homologous to *Bacillus* sp. α-amylase 406 and 75% to *Bacillus amyloliquefaciens* α-amylase. Given the high structural similarity of α-amylase molecules from various sources (e.g. Janeček, 1994, 1997), and in particular the liquefying *Bacillus* α-amylases (Yuuki, 1985), significant differences in toxicological properties between these homologous enzymes are not expected.

Alpha-amylases derived from both fungal and bacterial sources have a long history of safe use in the food industry (Olempska-Beer *et al.*, 2006). Alpha-amylase (as carbohydrase) from *A. niger* is recognized as GRAS (Generally Recognized As Safe) according to GRAS Notice 89, and α-amylase from *A. oryzae* is GRAS according to GRAS Notice 90. Alpha-amylase obtained from *B. licheniformis* has been affirmed as GRAS by the US FDA (as mixed carbohydrase and protease enzyme preparation (21CFR184.1027)). In addition GRAS Notices have been submitted to the US FDA for α-amylase obtained from genetically modified *B. licheniformis* strains, e.g. hybrid *B. licheniformis* / *B. amyloliquefaciens* α-amylase (GRN 22), *G. stearothermophilus* (formerly *called B. stearothermophilus*, GRN 24) α-amylase, modified *B. licheniformis* α-amylase (GRN 79). Based on the information provided in these GRAS Notices, the agency did not question the

conclusion that such α-amylase food enzyme preparations produced with *B. licheniformis* are GRAS under the intended conditions of use.
Various other countries also approved α-amylase preparations derived from *B. licheniformis*, e.g. Canada (see list of permitted enzymes), France (see Arrêté du 19 Octobre 2006), and Australia/New Zealand (α-amylase, see Australian Standard 1.3.3). Also JECFA approved α-amylase produced by *B. licheniformis* (JECFA 1987, 2004). Alpha-amylases produced by production organisms other than *B. licheniformis* have also been proven safe worldwide. For example, JECFA approved α-amylases from *Aspergillus niger* (JECFA, 1975, p. 124), *Aspergillus oryzae* (JECFA, 1988, p. 5), *B. megaterium* (JECFA 1991, p. 77), *B. subtilis* (JECFA 1991, p. 67), and *B. stearothermophilus* (JECFA 1991, p. 63, JECFA 1991, p. 71).

In Australia/New Zealand α-amylase from *A. niger*, *A. oryzae*, *B. amyloliquefaciens*, *B. subtilis* and *B. stearothermophilus* have been approved (Australian Standard 1.3.3).
Canada approved α-amylases from *A. niger*, *A. oryzae*, *B. amyloliquefaciens*, *B. subtilis*, *B. stearothermophilus*, *Rhizopus oryzae*, and Barley Malt (Canadian Food and Drug Regulation).

In Denmark α-amylases from *A. oryzae* and *B. amyloliquefaciens* have been approved, and in France α-amylases from *A. niger*, *A. oryzae*, *B. amyloliquefaciens*, *B. subtilis*, and *P. fluorescens* (Arrêté du 19 Octobre 2006).

4. MANUFACTURING PROCESS

This section describes the manufacturing process for the α-amylase enzyme which follows standard industry practice (Kroschwits, 1994; Aunstrup *et al.*, 1979; Aunstrup, 1979). For a diagram of the manufacturing process, see Appendix 3. The quality management system used in the manufacturing process complies with the requirements of ISO 9001. The enzyme preparation is manufactured in accordance with FDA's current Good Manufacturing Practices ("cGMP") as set forth in 21 C.F.R. Part 110.

4.1 Raw Materials

The raw materials used in the fermentation and recovery process for this α-amylase (C16F) are standard ingredients used in the enzyme industry (Kroschwits, 1994; Aunstrup *et al.,* 1979; Aunstrup, 1979). All the raw materials conform to the specifications of the Food Chemicals Codex (FCC), 10th edition (US Pharmacopeia, 2016), except for those raw materials that do not appear in the FCC. For those not appearing in the FCC, internal requirements have been set in line with FCC and JECFA requirements and acceptability of use for food enzyme production. DuPont industrial Biosciences uses a supplier quality program to qualify and approve suppliers. Raw materials are purchased only from approved suppliers and are verified upon receipt.

Glucose (which may be from wheat) and soy flour are used in the fermentation process, but both will be consumed by the microorganism as nutrients. The final C16F α-amylase enzyme preparation which is the subject of this GRAS notice does not contain any major food allergens from the fermentation medium.

4.2 Fermentation Process

The α-amylase enzyme (C16F) is manufactured by submerged fermentation of a pure culture of the genetically modified strain of *B. licheniformis* 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 conducted periodically to ensure absence of foreign microorganisms and confirm production strain identity.

4.2.1 Production organism

A new lyophilized stock culture vial of the *B. licheniformis* production organism described in Section 2 is used to initiate the production of each batch. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

4.2.2 Criteria for the rejection of fermentation batches

Growth characteristics during fermentation are observed microscopically. Samples are taken from each fermentation stage (inoculum, seed, and main fermentor) before inoculation, at regular intervals during growth and before harvest or transfer. These samples are tested for microbiological contamination by plating on a nutrient medium. If a fermentation batch is determined to be contaminated, it will be rejected if deemed necessary. If the contamination is minor and determined to be from common non-pathogenic environmental microbes, the fermentation may be processed.

4.3 Recovery Process

The recovery process is a multi-step operation, which starts immediately after the fermentation process.
The enzyme is recovered from the culture broth or ultra-filtered concentrate (UFC) by the following series of operations:

1) Primary separation –centrifugation or filtration;
2) Concentration – ultrafiltration;
3) Addition of stabilizers/preservatives;
4) Polish filtration

4.4 Formulation/Standardization

The ultra-filtered concentrate (UFC) enzyme preparation is stabilized by final formulation to contain ~ 0.1% sodium benzoate, ~ 0.5% potassium sorbate, ~ 9.0% sodium chloride and up to 33% glycerol at pH 6-6.5. The remaining is water.

5. COMPOSITION AND SPECIFICATIONS

5.1 Quantitative Composition

Ultra-filtered concentrate (UFC) enzyme preparation

The liquid concentrate is stabilized with the formulation ingredients listed below and tested to demonstrate that it meets the specifications. Various commercial formulations exist, with a range of enzyme activities. The following is a representative composition:

Enzyme Activity:	27150-31850 DLU/g
Sodium chloride	8.5-9.5%
Glycerol	27-33%
Potassium sorbate	0.4-0.6%
Sodium benzoate	0.1%
Remaining is water	
pH	6-6.5

The preparation includes TOS (total organic solids resulting from the fermentation) of approximately 9.09%.

5.2 Specifications

C16F α-amylase regardless of product format, meets the purity specifications for enzyme preparations set forth in the FCC 10th edition (2016). In addition, it also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by JECFA in the Compendium of Food Additive Specification (2006). The results of analytical testing of the 3 lots of product is given in Appendix 4 verifying that it meets FCC 10th edition (U.S. Pharmacopeia, 2016) and JECFA (2006) specifications for enzyme preparations as well as the absence of live production organisms.

6. APPLICATION

6.1 Mode of Action

The α-amylase functions in the endohydrolysis (1→4)-α-D-glucosidic linkage in polysaccharides containing three or more (1→4)-α-linked D-glucose units. It acts on starch, glycogen and related polysaccharides and oligosaccharides in a random manner; reducing groups are liberated in the α-configuration (the initial anomeric configuration of the free sugar group released).

6.2 Uses and Use Level

The C16F α-amylase will be used as a processing aid in carbohydrate processing, including starch hydrolysis to manufacture sweeteners (such as HFCS), specialty starches, and in fermentation to produce organic acids (i.e. lactic and citric acid), and potable alcohol. In all of these applications, the enzyme is not present or active in the final food or present in negligible amounts with no function in the final food.

6.2.1 Uses

The enzyme product will be used in the following applications:

Carbohydrate processing

The C16F α-amylase will be used in combination with other enzymes for the manufacture of glucose from granular starch from various sources including corn, wheat, milo, tapioca, barley, rice, potatoes and cassava. The resultant glucose-rich syrups can be purified to meet various specifications: crystallized to produce dextrose, isomerized to produce high fructose corn syrup.

The purification process for glucose and fructose syrups production will include carbon ion exchange (large local pH swings) and evaporation at temperatures up to 85°C for 30 minutes or less. Denatured enzyme ends up in co-products such as corn gluten feed/meal used in animal feed.

The α-amylase may also be used to treat liquefied starch for the manufacture of starch syrups with special saccharide distribution. The process will involve evaporation of the syrups, at temperatures up to 85°C for 30 minutes or less.

Potable Alcohol

The C16F α-amylase will be used in combinations with other enzymes (glucoamylases, proteases, etc.) to maximize the conversion of ground grain or other starchy substrate to fermentable carbohydrate. Yeast fermentation targets include potable alcohol and organic acids such as citric and lactic acid. After saccharification and fermentation are completed, the slurry goes through distillation at ~ 85° C. The water phase goes to evaporation and the solids go to dryers. Denatured enzyme ends up in the distillers' grains (used in animal feed).

6.2.2 Use Levels

The C16F α-amylase will be used in carbohydrate processing in the manufacture of high fructose corn syrup (HFCS), and in fermentation to produce potable alcohol, and organic acids (citric acid, lactic acid) for use in food.

The proposed application rate of the clarified C16F α-amylase is 5-6.2 mg total protein (TP) per kg of dry starch substance (worst case).

As noted above, the C16F α-amylase is expected to be inactivated or removed during the subsequent production processes for all applications. The enzyme is added during carbohydrate processing after the liquefaction step. After that, the glucose rich syrup or starch syrup obtained goes through several purification steps (filtration, carbon treatment, ion exchange, etc.), so no carryover of the C16F α-amylase is expected. In potable alcohol production, the alcohol is distilled after the C16F α-amylase is used, so the alcohol does not contain the α-amylase.

Residual enzyme protein (inactive) will be present in co-products used for animal feed such as distillers' grains (DG) and corn gluten meal, which are defined feed ingredients in the 2015 American Association of Feed Officials (AAFCO) Official Publication.

6.3 Enzyme Residues in the Final Foods

As noted above, the C16F α-amylase is expected to be inactivated or removed during the subsequent production processes for all applications. In the rare case that inactive α-amylase enzyme is present in the processed food and is ingested; it will not be absorbed intact. Instead, the enzyme is broken down by the digestive system into small peptides and amino acids, with the latter being absorbed and metabolized, which poses no human health risk.

7. SAFETY EVALUATION

7.1 Safety of the Production Strain

The safety of the production organism must be the prime consideration in assessing the safety of an enzyme preparation intended for use in food (Pariza and Foster, 1983). 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 (IFBC, 1990). Pariza and Foster (1983) define a non-toxigenic organism as 'one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure' and a non-pathogenic organism as 'one that is very unlikely to produce disease under ordinary circumstances.' *Bacillus licheniformis* strains used in enzyme manufacture meet these criteria for non-toxigenicity and non-pathogenicity.

7.1.1 Safety of the host organism

B. licheniformis is a known safe host for enzyme production and is widely used by enzyme manufacturers around the world for the production of enzyme preparations for use in human food, animal feed, and numerous industrial enzyme applications. *B. licheniformis* is considered a benign organism that does not possess traits that cause disease. This also applies to the DuPont Industrial Biosciences (legacy Genencor) *B. licheniformis* host strain, which has been demonstrated to be non-pathogenic, non-toxigenic and not cytotoxic.

The species *Bacillus licheniformis* is an accepted source of safe food enzymes in the literature. The safety of *B. licheniformis* strains was reviewed by De Boer *et al* (1994). Pathogenic strains are not described in the Bergey Manual or in the ATCC and other catalogues. The species *Bacillus licheniformis* does not appear on the EU Council Directive amending the "Directive 90/679/EEC on the protection of workers from risks related to exposure to biological agents at work". The species *B. licheniformis* is accepted as a safe host for the construction of Risk Group I GMMs in several countries, like Germany, The Netherlands, etc. and is exempted as a host under the NIH Guidelines in the USA. It is also on the Tier 1 exempt list used by the US EPA, exempting the species from standard notification requirements under the TSCA Biotechnology Rule.

The US Food and Drug Administration reviewed the safe use of food-processing enzymes from recombinant microorganisms, including *B. licheniformis* (Olempska-Beer *et al.* 2006). An extensive risk assessment of *B. licheniformis*, including its history of commercial use has been published by the US Environmental Protection Agency (1997). It was concluded that *B. licheniformis* is not a human pathogen nor is it toxigenic.

Mixed carbohydrase and protease preparation from *B. licheniformis* was affirmed as Generally Recognized as Safe (GRAS) for use as direct food ingredients in the US Code of Federal Register (21CFR184.1027). In addition, (GRAS) Notices have been submitted to the US FDA for several food enzymes from genetically modified *Bacillus licheniformis* strains, including pullulanase (GRN 72), α-amylase (GRN 22, GRN 24, GRN 79), glycerophospholipid cholesterol acyltransferase, GCAT (GRN 265), and maltotetraohydrolase (GRN 277). Based on the information provided in these GRAS Notices, the agency did not question the conclusion that food enzyme preparations from *B. licheniformis* are GRAS under the intended conditions of use.

In various countries enzyme preparations derived from *B. licheniformis* have been approved, e.g. Canada (α-amylase, protease and pullulanase, see Canadian Food and Drug Regulation), France (α-amylase, protease, pullulanase and cyclomalto-dextrine glucotransferase, see Arrêté du 19 Octobre 2006), and Australia/New Zealand (α-amylase, pullulanase, see Australian Standard 1.3.3). Also JECFA approved α-amylase produced by *B. licheniformis* (JECFA 1987, 2004).

The European Food Safety Agency (EFSA) maintains a list of the biological agents to which the Qualified Presumption of Safety (QPS) assessment can be applied. In 2007, the Scientific Committee set out the overall approach to be followed, and established the first list of the biological agents. The QPS list is reviewed and updated annually by the Panel on Biological Hazards (BIOHAZ). If a defined taxonomic unit does not raise safety concerns or if any possible concerns can be excluded, the QPS approach can be applied and the taxonomic unit can be recommended to be included in the QPS list. The safety of *B. licheniformis* as a production organism has been assessed by EFSA and been accorded QPS status provided the qualification requirements are met (see http://www.efsa.europa.eu/en/topics/topic/qps.htm?wtrl=01). For *Bacillus* strains the specific requirement is absence of toxigenic activity, which has been tested for the host strain.

B. licheniformis strains in general have been used for more than 20 years for the industrial production of α-amylase (de Boer *et al*, 1994). The strain *B. licheniformis* Bra7 and strains derived from it have been in use for industrial scale production of α-amylase for food processing applications since 1989, with food grade versions in use for grain processing since 1998.

7.1.2 Safety of the donor organism

The species used as a source for the α-amylase sequence is a *Cytophaga* sp., a soil bacterium described by Jeang *et al.* (1995) and Jeang *et al.* (2002). The Genus *Cytophaga* is described in the List of Prokaryotic names with Standing in Nomenclature (http://www.bacterio.net/cytophaga.html) as follows:

Cytophaga Winogradsky 1929, genus. (Type genus of the order Cytophagales Leadbetter 1974 [Approved Lists 1980]; type genus of the family Cytophagaceae Stanier 1940 [Approved Lists 1980]).

Type species: *Cytophaga hutchinsonii* Winogradsky 1929 (Approved Lists 1980).

Synonym: "Promyxobacterium" Imshenetski and Solntseva 1945.

Etymology: Gr. n. kutos, hollow, vessel, jar, and in biology a cell; Gr. v. phagein, to eat; N.L. fem. n. Cytophaga, devourer of cell; intended to mean devourer of cell wall, cellulose digester.

References: SKERMAN (V.B.D.), McGOWAN (V.) and SNEATH (P.H.A.) (editors): Approved Lists of Bacterial Names. *Int. J. Syst. Bacteriol.*, 1980, 30, 225-420 [WINOGRADSKY (S.): Études sur la microbiologie du sol - sur la dégradation de la cellulose dans le sol. Annales de l'Institut Pasteur (Paris), 1929, 43, 549-633.]

The recent mini review by Kirchman (2002) provides the following information:

Cytophaga-like bacteria are unicellular, gliding, nonspore-forming Gram-negative rods. They are part of the *Cytophaga-Flavobacteria* cluster, which are especially proficient in degrading various biopolymers such as cellulose, chitin, and pectin. They can be found in just about every habitat in the biosphere, including kusaya (a Japanese delicacy consisting of putrid fish), rumens, hydrothermal vents, rocks and sea-ice in Antarctica, and sediments of lakes and the oceans.

Cytophaga-Flavobacteria seems particularly common in the oceans. In fact, in many oceanic habitats, the *Cytophaga-Flavobacteria* cluster is the most abundant of all bacterial groups. However, the taxonomy of the *Cytophaga-Flavobacteria* cluster is problematic. The genus name *Cytophaga* is scattered throughout the entire *Bacteroidetes* phylogenetic tree. It may be needed to divide *Cytophaga* into several genera and even higher taxa.

The α-amylase from donor strain *Cytophaga* sp. has been described by Jeang *et al.* (1995 and 2002). Little has been described about the strain though, except that it is typed as a *Cytophaga* species and was isolated from soil. A literature search was performed on September 25, 2014 in SciFinder (combined CAS and Medline databases, on file with DuPont (Legacy Genencor) Product Stewardship and Regulatory (PS&R) using the search terms "Cytophaga" (2568 hits) in combination with terms "food safety or toxicity or pathogenicity", resulting in 92 records of interest. A review of the abstracts revealed that some members of the genus are reported to be fish pathogens (Carson *et al.*, *Journal of Fish Diseases* 16:209-218, 1993). However, pathogenicity is a complex process that typically involves the expression of specialized invasive elements called virulence factors, none of which are associated with the α-amylase protein or its gene. Many harmless microorganisms express genes for amylases, which are used in numerous industrial applications including food manufacture (Pandey *et al.*, *Biotechnol. Appl. Biochem.* 31:135–152, 2000). The only genetic information expressed in the production host is a synthetic α-amylase variant gene inspired from the *Cytophaga* sp. α-amylase sequence, but no actual *Cyptophaga* sp. DNA was transferred.

7.2 Safety of the Manufacturing Process

The manufacturing process for the production of C16F α-amylase will be conducted in a manner similar to other food and feed production processes. It consists of a pure-culture fermentation process, cell separation, concentration and formulation, resulting in a liquid α-amylase enzyme preparation. The process, described in Appendix 3, is conducted in accordance with Good Manufacturing Practice (GMP) as set forth in 21 CFR Part 110. The resultant product meets the purity specifications for enzyme preparations of the Food Chemicals Codex, 10th Edition (US Pharmacopeia, 2016) and the general specifications for enzyme preparations used in food processing proposed by FAO/WHO (JECFA, 2006). The final C16F α-amylase enzyme preparation which is the subject of this GRAS notice does not contain any major food allergens.

7.3 Safety of *Bacillus licheniformis* α-amylase

The C16F α-amylase amino acid sequence was modified in 7 amino acid positions. However, the resultant variant sequence does not differ in size or safety profile from the native amylase enzyme. The variant C16F α-amylase sequence was assessed for toxin and allergen potential as outlined below.

7.3.1 Toxin homology

A Basic Local Alignment Search Tool (BLAST) search for homology of the mature C16F amino acid protein sequence below with known toxins and antinutrients was performed using the UniProtKB annotated Protein Knowledge database (Magrane *et al.*, 2011; http://www.uniprot.org/), UniProt release 2015_05 (April 29, 2015). This database contains 549008 proteins (http://web.expasy.org/docs/relnotes/relstat.html), of which 5577 are manually annotated as toxins (http://www.uniprot.org/program/Toxins) and 6092 as venom proteins (http://www.uniprot.org/uniprot/?query=annotation%3A%28type%3A%22tissue+specificity%22+venom%29&sort=score).

From this search the top 1,000 hits in the UniProt database were exported to MS Excel, with the appropriate annotation fields (protein name, key words, gene ontology, protein family), allowing for use of search terms "toxin" and "venom". Results show that the vast majority of hits were with α-amylases with none of the top 1,000 database hits annotated as either toxin or venom.

7.3.2 Allergenicity

The most current allergenicity assessment guidelines developed by the Codex Commission (2009) and Ladics *et al.* (2011) recommend the use of FASTA or BLASTP search for matches of 35% identity or more over 80 amino acids of a subject protein and a known allergen. Ladics *et al.* (2011) further discussed the use of the "E-score or E-value in BLAST algorithm that reflects the measure of relatedness among protein sequences and can help separate the potential random occurrence of aligned sequences from those alignments that may share structurally relevant similarities." High E-scores are indicative that any alignments do not represent biologically relevant similarity, whereas low E-scores ($<10^{-7}$) may suggest a biologically relevant similarity (i.e., in the context of allergy, potential cross reactivity). They suggest that the E-score may be used in addition to percent identity (such as > 35% over 80 amino acids) to improve the selection of biologically relevant matches. The past practice of conducting an analysis to identify short, six to eight, contiguous identical amino acid matches is associated with false positive results and is no longer considered a scientifically defensible practice.

The Codex Commission states:
"A negative sequence homology result indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known allergens."

Appendix 2 lists the *Cytophaga sp.* α-amylase variant sequence in FASTA format, without its secretion signal.

The search for 80-amino acid stretches within the sequence with greater than 35% identity to known allergens using the Food Allergy Research and Resource Program (FARRP) AllergenOnline database (http://www.allergenonline.org/index.shtml) containing 1956 (version released Jan 27, 2016) peer-reviewed allergen sequences (listed in http://www.allergenonline.org/databasebrowse.shtml) reveals no matches to known allergens.

Full FASTA alignment of the above sequence with known allergens using E-value <0.1 as the cut-off revealed one match with an E-score[1] of $3.2x10^{-4}$ and an identity of 23.8% (NCBI gi|94706935|sp|POC1B3.1|AMYA1_ASPOR), which corresponds to TAKA amylase-A, an α-amylase from *A. oryzae*, also referred to as Asp o 21. However, full sequence FASTA alignment is recommended specifically to support any positive findings in the codex 80 amino acid/35% criteria. Hence, by itself it does not indicate sufficient homology specially, at a relatively high E-value exceeding 10^{-5}.

Since the two enzymes, *Cytophaga sp.* α-amylase and TAKA-amylase A, are both α-amylases, some homology is not surprising, even across fungal and bacterial amylases.

Although cautioned against by Ladics *et al.* (2011) and even in the Codex (2009) guidelines, and as further elaborated on AllergenOnline.org that there is no evidence that an 8 amino acid match will identify a protein that is likely to be cross-reactive and could be missed by the conservative 80 amino acid match (35%), this database does allow for isolated identity matches of >8 contiguous amino acids to satisfy demands by some regulatory authorities for this extremely precautionary search. Performing this search produced no matches with known allergens.

In conclusion, based on the sequence homology alone, (no match with either codex criterion) the α-amylase variant enzyme, C16F amylase, from *Cytophaga* sp. is unlikely to pose a risk of food

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[1] The AllergenOnline database help page (http://www.allergenonline.org/databasehelp.shtml) states:
"For a database the size of AllergenOnline, two sequences might be considered related in evolutionary terms (i.e. diverged from a common ancestor and share common three-dimensional structure), when the E-value of the FASTA query is less than 0.02 (Pearson, 1996). However, a value of 0.02 does not mean that the overall structures are likely to be sufficiently similar for antibodies (e.g. IgE from an allergic individual) against one protein to recognize the other. To identify proteins that may share immunologic or allergic cross-reactivity, matches with E-values larger than 10^{-7} are not likely to identify relevant matches, while matches with E-values smaller than 10^{-30} are much more likely to be cross-reactive in at least some allergic individuals (Hileman, 2002). Since E-values depend to a great degree on the scoring matrix, the size of the database and many other factors, interpretation of immunological significance should be viewed with caution. As such, it is recommended to use a conservative E score value (e.g. 10^{-7}) as an additional data point to complement the percent identity score.

allergenicity. As for all enzyme products, the MSDS for the α-amylase product includes a precautionary statement that inhalation of enzyme mist/dust may cause allergic respiratory reactions, including asthma, in susceptible individuals on repeated exposure.

7.3.3 Safety of use in food

In addition to the allergenicity assessment described above, the safety of the C16F α-amylase has also been established using the Pariza and Johnson (2001) decision tree:

1. Is the production strain[1] genetically modified?[2,3]

Yes, Go to 2.

2. Is the production strain modified using rDNA techniques?

Yes. Go to
3a.

3a. Does the expressed enzyme product which is encoded by the introduced DNA[4,5] have a history of safe use in food[6]?

Yes, α-amylase has been used for years in food processing. Although the *Cytophaga* sp. α-amylase (C16F) is new as an isolate in food processing, the variant α-amylase expressed in *Bacillus licheniformis* is still an α-amylase with the designation IUBMB 3.2.1.1. Given the high sequence similarities of CF16 α-amylase to α-amylase molecules from various sources (e.g., 81% identity with α-amylase from *Bacillus* sp. 406 and 75% identity with α-amylase from *Bacillus amyloliquefaciens*), C16F α-amylase is considered substantially equivalent to these α-amylases with extensive history of safe use. US FDA affirmed the GRAS status of mixed carbohydrase/protease enzyme preparation derived from *B. licheniformis* and α-

[1] Production strain refers to the microbial strain that will be used in enzyme manufacture. It is assumed that the production strain is nonpathogenic, nontoxigenic, and thoroughly characterized; steps 6–11 are intended to ensure this

[2] The term "genetically modified" refers to any modification of the strain's DNA, including the use of traditional methods (e.g., UV or chemically-induced mutagenesis) or rDNA technologies.

[3] If the answer to this or any other question in the decision tree is unknown, or not determined, the answer is then considered to be NO

[4] Introduced DNA refers to all DNA sequences introduced into the production organism, including vector and other sequences incorporated during genetic construction, DNA encoding any antibiotic resistance gene, and DNA encoding the desired enzyme product. The vector and other sequences may include selectable marker genes other than antibiotic resistance, noncoding regulatory sequences for the controlled expression of the desired enzyme product, restriction enzyme sites and/or linker sequences, intermediate host sequences, and sequences required for vector maintenance, integration, replication, and/or manipulation. These sequences may be derived wholly from naturally occurring organisms or incorporate specific nucleotide changes introduced by *in vitro* techniques, or they may be entirely synthetic.

[5] If the genetic modification served only to delete host DNA, and if no heterologous DNA remains within the organism, then proceed to step 5.

[6] Engineered enzymes are considered *not* to have a history of safe use in food, unless they are derived from a safe lineage of previously tested engineered enzymes expressed in the same host using the same modification system.

amylase and β-glucanase from *B. amyloliquefaciens* for use in food with GMP as the only limitation (21CFR 184.1027 and 1148, respectively). In addition α-amylases from several genetically modified *B. licheniformis* strains were GRAS notified to FDA, including hybrid *B. licheniformis / B. amyloliquefaciens* α-amylase (GRN 22), and modified *B. licheniformis and B. amyloliquefaciens* α-amylase (GRN 79), and the agency issued "no questions" letters in response. The safety of C16F α-amylase is further supported by its lack of sequence similarity with known food allergens and oral toxins.

Go to 3c.

3c. Is the test article free of transferable antibiotic resistance gene DNA[1]?

Yes. No transferable antibiotic resistance gene DNA is present in the enzyme preparation. Go to 3e.

3e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products? Yes, inserted DNA is well characterized and free of unsafe attributes. Go to 4.

4. Is the introduced DNA randomly integrated into the chromosome?

No, as it is integrated at the *cat* locus. Go to 6.

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure[2]?

Yes. The *B. licheniformis* Bra 7 safe lineage is established as discussed in Appendix 5. Its safety as a production host and methods of modification are well documented and their safety have been confirmed through repeated toxicology testing (see Appendix 5). The established NOAEL is sufficient to support the intended uses.

Conclusion: Article is accepted.

Based on the publicly available scientific data from the literature and additional supporting data generated by DuPont, the company has concluded that α-amylase from *Bacillus licheniformis,* JML1584 is safe and suitable for use in carbohydrate processing including the

[1] Antibiotic resistance genes are commonly used in the genetic construction of enzyme production strains to identify, select, and stabilize cells carrying introduced DNA. Principles for the safe use of antibiotic resistance genes in the manufacture of food and feed products have been developed (IFBC, 1990; "FDA Guidance for Industry: Use of Antibiotic Resistance Marker Genes in Transgenic Plants (http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Biotechnology/ucm096135.htm)

[2] In determining safe strain lineage one should consider the host organism, all of the introduced DNA, and the methods used to genetically modify the host (see text). In some instances the procedures described by Pariza and Foster (1983) and IFBC (1990) may be considered comparable to this evaluation procedure in establishing a safe strain lineage.

manufacture of sweeteners such as high fructose corn syrup (HFCS), and fermentation to produce organic acids (citric acid, lactic acid) and potable alcohol. Further, the α-amylase is Generally Recognized as Safe (GRAS) for those uses.

Although the Pariza and Johnson evaluation resulted in the conclusion to accept the enzyme preparation as safe without new toxicology testing, the safety of C16F enzyme preparation was further confirmed through unpublished toxicological testing as described below. The toxicology testing was conducted to be able to use the results in countries where toxicology testing is required for enzyme preparation approval. In addition, we also conducted toxicology studies with C16F enzyme preparation from which the inactivated production organism was not removed (data not shown).

7.3.4 Safety Studies

Dupont Industrial Biosciences (legacy Genencor) has performed many studies on the toxicity of *B. licheniformis* α-amylase, both the wild type and protein-engineered variants. Also toxicity studies on *B. stearothermophilus* α-amylase from its natural and recombinant sources have been performed by Dupont/Genencor and others (MacKenzie *et al*, 1989). These studies also serve to demonstrate the safety of the Bra7 homologous based host strain used here.

This is accomplished through testing of a low pH, oxidatively stable, α-amylase preparation by completing a 28-day Oral Toxicity Study in Rats, an Acute Oral Toxicity Study in Rats, a Bacterial Reverse Mutation Assay, an *in vitro* Mammalian Cytogenetic Test Using Human Peripheral Lymphocytes, a *Salmonella-Escherichia coli*/Mammalian Microsome Reverse Mutation Assay with Confirmatory Assay, and a Chromosome Aberration test in Human Peripheral Blood Lymphocytes. In addition, the stable α-amylase heterologous production organism and its non-recombinant host were also studied in an Acute Toxicity/Pathogenicity Study in Rats.

Lastly, the host strain Bra7 itself was tested for *Bacillus* toxin production (enterotoxins or emetic toxins) in the CHO-MTT cytotoxicity screening test (Mossman, 1983) as recommended in the "Opinion of the Scientific Committee on Animal Nutrition on the Safety of use of *Bacillus* species in animal nutrition", published by the European Commission Health and Consumer Protection Directorate General (17 February 2000).

All studies demonstrated that the α-amylase products produced by the Bra7 based host strains are safe for their intended use and that the pathogenic/toxigenic potential of the production organism was no different from that of the non-recombinant host.

DuPont has determined by scientific procedures that production organism *B. licheniformis* used by legacy Genencor (now DuPont Industrial Biosciences) is derived from a safe strain lineage. A review of numerous toxicology studies conducted with enzyme preparations produced by different strains of *B. licheniformis* indicates that, regardless of the production organism strain, all enzyme preparations are: not irritating to the skin and eyes, not skin sensitizers, not mutagenic or clastogenic in genotoxicity assays and do not adversely affect any specific target organ. Due to the consistency of the findings from enzyme preparations derived from different *B. licheniformis* strains, it is expected that any new enzyme preparation produced from *B. licheniformis* strains would behave similarly from a toxicological standpoint.

Three toxicology studies (90-day oral gavage study, a chromosomal aberration study, and an Ames assay) were conducted with C16F α-amylase ultra-filtrate concentrate (UFC) from *B. licheniformis* JML1584 have been completed, in order to satisfy certain national regulatory approval requirements outside the US, the results of which further support this GRAS determination.

The results are evaluated, interpreted and assessed in this document. The test material, an ultra-filtrate concentrate (UFC) was used in the aforementioned 3 toxicology studies, having the following characteristics:

Lot No.:	20138088 UFC
Physical:	Fermentation liquid, brown
Enzyme activity:	68298 amylase DLU/ml
pH:	6.3
Specific gravity:	1.03 g/ml
Total protein:	39.8 mg/ml
TOS:	7.12 %

All safety studies were conducted in accordance with internationally accepted guidelines (OECD) and are in compliance with the principles of Good Laboratory Practices ("GLP") according to the FDA/OECD.

Study summaries are included below:

Toxicology studies- C16F α-amylase UFC

1) Bacterial Reverse Mutation Assay – Ames assay (BioReliance, Study No. H-30929, 2014)

This assay was conducted in accordance with OECD guideline No. 471 (1997)

a. Procedure

The objective of this assay was to assess the potential of α-amylase (C16F UFC) to induce point mutations (frame-shift and base-pair) in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and *Escherichia coli* strain WP2 *uvrA*. The test material was tested both in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). The assay was performed in two phases using the plate incorporation methodology for the positive control, 2-aminoanthracene, with *E. coli* and the treat and plate methodology for the all remaining strains and assays. A screening (dose range) test was performed first to select the dose levels for the confirmatory assay. Vehicle control, positive control and 8 doses of the test article were plated, two plates per dose, with overnight cultures of all four strains of *Salmonella typhimurium* and *E. coli* WP2 *uvr*A in the presence and absence of S-9 mix. In the confirmatory assay, 6 doses of the test article along with appropriate vehicle and positive controls were plated in triplicate in the presence and absence of S-9 mix. All dose levels were expressed in terms of total protein (TP). The highest dose level tested was 5000 μg TP/plate, which is the maximum dose required by the OECD guideline. The positive controls used for assays without S-9 mix were 2-nitrofluorene, N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and ICR-191. For assays with S-9 mix, the positive control was 2-aminoanthracene. Vehicle control plates were treated by the addition of sterile deionized water.

b. Results
In the screening assay, α-amylase (C16F UFC) was toxic to strain TA98 in the absence of S-9 mix at 5000 μg TP/plate. It is not toxic to all other test bacteria up to and including the highest dose level tested (5000 μg TP/plate) in both the absence and presence of S-9 mix. No positive mutagenic responses were observed with any of the tester strains in the presence and absence of S-9mix. Based on the findings of the screening assay, 5000 μg TP/plate was selected as the highest dose level for the confirmatory assay. In the confirmatory assay, six dose levels (15, 50, 150, 500, 1500, and 5000 μg TP/plate) were tested. Precipitate was not observed. Toxicity was noted only in strain TA98 at 5000 μg TP/plate in the absence of S-9 mix. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of metabolic activation. Statistical increases in the number of revertant colonies were noted with the positive controls in both the presence and absence of metabolic activation substantiating the sensitivity of the treat and plate assay and the efficacy of the metabolic activation mixture.

c. Evaluation
Under the conditions of this assay, α-amylase (C16F UFC) has not shown any evidence of mutagenic activity in the Ames assay in both presence and absence of metabolic activation.

2) *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes. (DuPont Haskell Global Centers, Study No. H-30929, 2014).

This assay was conducted in accordance with OECD guideline No. 473 (1977).

a. Procedure
The objective of this assay was to investigate the potential of α-amylase (C16F UFC) to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). In this assay, human lymphocytes were stimulated to divide by the

addition of a mitogen (e.g., phytohemagglutinin, PHA). Mitotic activity began at about 40 hours after PHA stimulation and reached a maximum at approximately 3 days.
Alpha-amylase (C16F UFC) was mixed with cultures of human peripheral lymphocytes both in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver; S-9 mix). This assay consisted of a preliminary toxicity (dose range finding) assay and two main assays. In the preliminary assay, all cultures with or without S-9 mix were treated for 4 hours and continuously for 22 hours in the absence of S-9 mix. All cells were harvested 22 hours after treatment initiation. Nine concentrations of α-amylase (C16F UFC) ranging from 50 to 5000 μg TP/ml were used and at least 5 dose levels were then selected for the definitive assay with the highest dose level clearly inducing a toxic effect (50% reduction in mitotic index). Cytotoxicity is characterized by the percentage of mitotic suppression in comparison to the controls. In the absence of cytotoxicity, the highest dose selected would be 5000 μg TP/ml, as recommended by the OECD guideline. All dose levels were expressed in terms of total protein.

In the definitive assay, cultures with and without S-9 mix were exposed to the test article for 4 hours, and continuously for 22 hours in the absence of S-9 mix. Cells were collected 22 hours (1.5 normal cell cycles) after initiation of treatment. Two hours prior to harvest, Colcemid was added to the cultures at a final concentration of 0.1 μg/ml to arrest mitosis.

Cells were collected by centrifugation, treated with 0.075 M KCl, washed with fixative, capped and stored overnight or longer. To prepare slides, the cells were re-suspended in fixative and then collected by centrifugation. The suspension of fixed cells was applied to glass microscope slides and air-dried. The slides were stained with Giemsa, permanently mounted and scored.

i. The mitotic index was recorded as the percentage of cells in mitosis per 500 cells counted. From these results, a dose level causing a decrease in mitotic index of 50% was selected as the highest dose in the main assays.
ii. Metaphase analysis (i.e., evaluation of chromosomal aberration) was conducted on at least 200 metaphases for each dose level (100 per duplicate treatment).
iii. Cells were scored for both chromatid-type and chromosome-type aberrations.
iv. Mitomycin C and cyclophosphamide were used as positive controls for cultures without S9 and cultures with S9, respectively.

b. Results
No visible precipitation of the test material in the culture medium was observed in cells exposed to 4 hours in both the presence and absence of S-9 mix. Substantial toxicity (at least 50% reduction in mitotic index relative to the vehicle control) was observed in the 22-hour non-activated test condition at concentrations greater than 100 μg/ml. Based on those findings, the highest concentration chosen was 5000 μg TP/ml for the 4-hour exposure condition (with and without S-9 mix) and 100 μg TP/ml for the 22-hour exposure condition (without S-9 mix).

In the definitive assay, the concentrations chosen for the 4-hour exposure (with and without S-9 mix) ranged from 250 to 5000 µg TP/ml. For the 22-hour exposure period (without S-9 mix), the concentrations chosen were 10, 25, 50, 75, and 100 µg TP/ml.
No test substance precipitation was observed. Substantial toxicity (at least 50% reduction in mitotic index relative to the vehicle control) was observed in the 22-hour exposure period (non-activated) at 100 µg TP/ml. Selection of doses for microscopic analysis was based on test substance induced toxicity in the 22-hour test condition. In the 4-hour test condition (with and without S-9 mix), selection of doses for microscopic analysis was based on the highest dose tested, 5000 µg TP/ml. Cytogenetic evaluations were conducted at 1000, 2500 and 5000 µg TP/ml in the 4-hour test conditions and at 25, 50 and 100 µg TP/ml in the 22-hour test condition. The test article did not induce any statistically significant increases in the frequency of cells with aberrations in either the presence or absence of S-9 mix. No increase in polyploidy metaphases was noticed. Significant increases in aberrant metaphases were demonstrated with the positive controls demonstrating the sensitivity of the tests and the efficacy of the S-9 mix.

c. Evaluation
Under the conditions of this test, α-amylase (C16F UFC) did not induce chromosomal aberrations (both structural and numerical) in this *in vitro* cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation up to the highest concentration 5000 µg TP/ml recommended by guidelines. All of the vehicle control cultures had frequencies of cells with chromosomal aberrations within the expected range. The positive control items inducted statistically significant increases in the frequency of cells with aberrations.

3) A 13-week Oral (Gavage) Toxicity Study in CD Rats. (MPI Research, Study No. H-30929, 2014).

This study was conducted in accordance with OECD guideline No. 408 (September 1998).

a. Procedure
The objective of this study was to investigate the potential of α-amylase (C16F UFC) to induce systemic toxicity after repeated daily oral administration to Charles River CD rats of both sexes for 90 continuous days. Groups of 10 animals per sex were treated by oral gavage with 0 (distilled water), 100, 250 or 500 mg TOS/kg bw/day. The dosing volume was 10 ml/kg bw/day. Animals of the same sex were housed in groups of two to three in solid floor polypropylene cages with stainless steel mesh lids and softwood bedding (non-aromatic) with access to water via an automatic system and feed *ad libitum*. For environmental enrichment, the animals were provided a supply of wooden chew blocks and cardboard fun tunnels. All groups were housed under controlled temperature, humidity and lighting conditions. All animals were observed daily for mortality and signs of morbidity. Body weight and feed consumption were recorded weekly. Water consumption was recorded twice weekly for each cage. Ophthalmologic examination was performed on all animals prior to study initiation and in the control and high dose groups at study

termination. Urinalysis, clinical chemistry and hematology were conducted at study termination. A functional observation battery consisting of detailed clinical observation, reactivity to handling and stimuli and motor activity examination was conducted during week 12 for the control and all treated groups. All animals were sacrificed at the end of the 13-week study. After a thorough macroscopic examination, selected organs were removed, weighed and processed for future histopathologic examination. Microscopic examination was initially conducted on selected organs from control and high dose animals.

b. Results

No treatment-related deaths were noted during the 13-week period. There were no treatment-related changes in body weights, feed consumption and water intake. Hematology and clinical chemistry conducted after 13 weeks of treatment did not reveal any adverse effects. There were no biological or statistical differences between the control and treated groups with respect to clinical observation, feed consumption, water consumption, ophthalmologic examinations, body weights, and body weight gains. There were no treatment-related changes in hematology and clinical chemistry at the end of week 13. There were no differences in the functional observation battery, grip strength and locomotor activity assays between treated and control animals. At necropsy, there were no treatment related findings on organ weights, macroscopic findings and histopathologic examinations. All microscopic findings were considered to be within the background incidence of findings reported in this age and strain of laboratory animals.

c. Evaluation and conclusion

Daily administration of α-amylase (C16F UFC) by oral gavage to CD rats at doses of 0, 100, 250 or 500 mg TOS/kg bw/day for 90 consecutive days did not result in treatment-related effects on clinical observations, feed consumption, body weight changes, hematology, clinical chemistry, urinalysis, organ weights, functional observation, grip strength or locomotor activities. No macroscopic or microscopic changes could be attributed to treatment. Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established at the highest dose tested, 500 mg TOS/kg bw/day (corresponding to 272 mg TP/kg bw/day).

CONCLUSION

The safety of α-amylase (C16F UFC) was assessed in a battery of toxicology studies investigating its genotoxic and systemic toxicity potential. Under the conditions of the mutagenicity assays α-amylase (C16F UFC) is not a mutagen or clastogen. Daily administration of α-amylase (C16F UFC) by gavage for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at 500 mg TOS/kg bw/day (corresponding to 272 mg TP/kg bw/day).

7.4 Safety Assessment

7.4.1 Identification of the NOAEL and allowable daily intake

In the 90-day oral (gavage) study in CD rats, a NOAEL was established at 272 mg total protein/kg bw/day (equivalent to 500 mg TOS/kg bw/day). The study was conducted in compliance with both the UK and OECD Good Laboratory Practice Regulations and was designed based on OECD guideline No. 408. Since human exposure to C16F α-amylase is through oral ingestion, selection of this NOAEL is thus appropriate.

No Observed Adverse Effect Level = 272 mg total protein/kg bw/day (UFC)
= 500 mg TOS/kg bw/day

Establishment of a Provisional Allowable Daily Intake (pADI)

Based on the results of the 90-day oral (gavage) study cited above, the NOAEL was established at 272 mg TP/kg/day (UFC). Application of a 100X margin of safety (10X for interspecies and 10X for intraspecies) to the NOAEL results in:

$$\text{pADI (UFC)} = \frac{\text{500 mg TOS/kg bw/day}}{\text{Safety factor (100)}} = \text{5.0 mg TOS/kg/day}$$

7.4.2 Human Exposure to C16F α-amylase

Uses and Applications

Alpha-amylase is used in grain/starch processing for production of fermentation products (potable alcohol, organic acids), sugar syrups, and starches with specialty saccharide distribution.

The dose rate inn fermentation and starch hydrolysis are set to be the same. The maximum application rate of this α-amylase is 6.2 mg protein/kg starch (11.09 mg TOS/kg starch). Process yield for alcohol and organic acids are set to be the same (35%); and for starch hydrolysis into sugar syrups and specialty starches, 100%. Exposure to CF16 α-amylase via potable alcohol, organic acids, sugar syrups and specialty starches is outlined below via the Budget Method.

The resulting maximum theoretical concentration of TOS from C16F α-amylase is 11.09 mg TOS/kg in specialty starches and sugar syrups and 31.69 mg TOS/kg for fermentation products (potable alcohol, organic acids).

Alpha-amylase in Grain/Starch processing for fermentation products (potable alcohol, organic acids) and starch hydrolysis products (sugar syrups, specialty starches)

	Potable alcohol, Organic acids (e.g., citric)	Sugar syrups Specialty starches
Dose (DLU/kg starch)	10639	
Dose (mg protein/kg starch)	6.2	
Dose (mg TOS/ kg starch)	11.09	
Yield, %	35	100
Concentration (mg TOS/kg)	31.69	11.09

A theoretical concentration of 31.69 mg TOS / L pure alcohol would result in exposure to TOS of 4.12 mg TOS for an adult consuming a maximum daily volume of 130 mL pure alcohol.

Concentration (mg UFC TOS/L, Alcohol, Organic acids)	31.69
Exposure Alcohol (mg UFC TOS/130 mL)	4.12

For a complete assessment of human exposure, the Budget method is used. This method was previously used by JECFA (FAO/WHO, 2001)[1] and accounts for exposure via liquid and solid foods.

Liquid Foods

Syrups and sweeteners are mostly applied in soft drink beverages and are therefore considered to be part of the category of liquid foods. Soft drinks typically contain 10-14% w/v HFCS so on average 120 g HFCS per L. In addition, the same soft drinks may contain small amounts or organic acid, e.g., 0.13% citric acid. Therefore, a final concentration of TOS from C16F α-amylase (via sugar syrups and citric acid) in beverages can be calculated as shown in the table below.

	Organic acids	Sugar syrups
Concentration (mg TOS/kg)	31.69	11.09
Ingredient concentration in beverages, %	0.13%	12%
TOS concentration in beverages (mg /L)	0.0412	1.331
Total TOS in beverages (mg / L)	1.372	
Intake of Liquids (mL/kg BW)	100	
Exposure via liquid intake (mg TOS/kg BW)	0.137	

[1] The Budget Method (Douglas, 1997) uses the following assumptions: For solid foods, the daily intake is set at 25 g/kg bw based on a maximum lifetime energy intake of 50 Kcal/kg bw/day. For liquid foods (non-milk beverages), a daily consumption of 100 ml/kg bw is used corresponding to 6 liters per day for a 60 kg adult. The concentration of enzyme in foods and beverages is the maximum application rate. Proportion of foods and beverages that contain the enzymes is set at 100%. The theoretical maximum daily intake (TMDI) is the sum of the TMDI for solid foods combined with the TMDI for beverages.

For the purpose of selecting an overall maximum exposure via liquids, the worst case TOS concentration from sugar syrups and organic acids combined (1.37 mg TOS mg/L) is appropriate, because:

- In distilled spirits the actual TOS concentration will be minimal compared to the maximum theoretical TOS concentration, as the enzyme protein and other organic solids will be removed in the distillation step.
- The maximum intake of any alcoholic drink will be limited largely by the maximum intake of alcohol the body can tolerate, not by the volume of the drink.

Hence, the higher exposures from sugar syrups and organic acids were used in our risk assessment to represent worst case scenario exposures via intake of liquids regardless of whether this is from consumption from soft drink or distilled spirits, with the assumption that 100 % of all consumed beverages are manufactured from grist treated with the α-amylase, resulting in an exposure estimate of 0.137 mg TOS/kg bw via liquid foods.

Solid Foods

This α-amylase is used in grain/starch processing in the manufacture of high fructose corn syrup (HFCS), sweeteners and modified starch which will then be used in bread, confectionary, and dairy. The estimated yield of both syrup and specialty starch from use of C16F AA is 100%. The yield in fermentation is 35%, with the resulting organic acids used at up to 2% in solid foods (e.g., citric acid in tart foods, sour candies, and lactic acid in meat processing). The calculations below assume that the diet of an adult contains about 40% starches and sugars. The exposure via sugar syrups and specialty starches far outweighs that via citric acid and lactic acid and thus via the Budget method we arrive at the following estimate of TOS intake from C16F AA:

	Organic acids (citric acid in tart foods, sour candy; lactic acid in meats)	Sugar syrups & specialty starches
Concentration (mg TOS/kg ingredient)	31.69	11.09
Ingredient concentration in solid food, %	2%	40%
TOS concentration in solid food (mg /kg)	0.634	4.436
Total TOS in solid food (mg /kg)	4.44	
Total Solids Intake (g/kg BW)	25	
Exposure via solid food (mg TOS/kg BW)	0.111	

Based on application rate, knowledge of process parameters, and using the budget method, the resulting theoretical exposure to α-amylase is based on calculation of Theoretical Maximum Daily Intake (TMDI) for liquid and solid foods:

TMDI for liquid foods:	0.137 mg TOS/kg bw/d
TMDI for solid foods:	0.111 mg TOS/kg bw/d
<u>TMDI Total:</u>	0.137 + 0.111 = 0.248 mg TOS/kg bw/day

Determination of the margin of safety

The margin of safety is calculated by dividing the NOAEL obtained from the 90-day oral (gavage) study in rats by the human exposure (worst case scenario). If the margin of safety is greater than 100, it suggests that the available toxicology data support the proposed uses and application rates.

$$\text{Margin of Safety} = \frac{\text{NOAEL (mg/kg/day) from applicable 90-day oral tox}}{\text{Human cumulative exposure (mg/kg/day)}}$$

$$\text{Margin of Safety} = \frac{500 \text{ mg TOS/kg bw/day}}{0.248 \text{ mg TOS/kg bw/day}}$$

$$= 2016$$

Please note that the calculated safety margin is the result of the maximum theoretical daily intake calculations resulting from the worst-case scenario that:

- all starch is processed with C16F AA, with all C16F AA carried over into resulting sugar syrups, specialty starches, and organic acids
- utilizing the Budget Method without adjustment factors, i.e., assuming that 100% of all liquid and solid food contain ingredients processed with C16F AA.

CONCLUSION

The safety of *Cytophaga* sp. α-amylase C16F expressed in *B. licheniformis* strain JML1584 as a processing aid in carbohydrate processing and potable alcohol production at the maximum recommended application rates is supported by existing toxicology data. The margin of safety for use of UFC derived C16F α-amylase in manufacture of fermentation products, syrups and specialty starches is calculated as 2016 based on a NOAEL of 500 mg TOS/kg bw/day (obtained from the cumulative maximum daily exposure to α-amylase C16F of 0.248 mg TOS/kg bw/day). In the rare case of ingestion of the α-amylase enzyme preparation, it poses no safety or health concerns to humans, based on maximum recommended application rates which are supported by existing toxicology data for this enzyme. Based on a margin of safety greater than 100 even in the worst-case, the uses of α-amylase as a processing aid in carbohydrate processing and production of organic acids and potable alcohol production are not of human health concern.

Note manufacture of potable alcohol and organic acids involves extensive purification of the end-products after fermentation. These process conditions enable the use of an alternative C16F preparation that is known as whole-broth amylase (C16F enzyme preparation from which the inactivated production organism is not removed). Toxicology studies separately performed with a test article based on whole-broth C16F preparation have indicated no adverse effects in the 90-day oral toxicity study up to the same top dose (500 mg TOS/kg bw) and no positives in the genotoxicity studies (Ames assay and chromosomal aberration). Notwithstanding that the preferred enzyme product form most applications is the clarified enzyme preparation prepared from UFC, the identical NOAELs for UFC and whole-broth C16F α-amylase preparations imply that the basic safety determination applies to both types of enzyme preparation containing C16F α-amylase.

8. BASIS FOR GENERAL RECOGNITION OF SAFETY

As noted in the Safety sections above, *B. licheniformis* and enzyme preparations derived from this production organism, including α-amylase, maltogenic α-amylase, pullulanase, subtilisin, and xylanase enzyme preparations, are well recognized by qualified experts as being safe. Published literature, government laws and regulations, reviews by expert panels such as JECFA, as well as DuPont Industrial Biosciences' own published and unpublished safety studies and GRAS determinations, support such a conclusion.

B. licheniformis is widely used by enzyme manufacturers around the world for the production of enzyme preparations for use in human food, animal feed, and numerous industrial enzyme applications. It is a known safe host for enzyme production.

Analysis of the safety based on the Pariza and Johnson (2001) decision tree indicates that *Cytophaga* sp. α-amylase expressed in *B. licheniformis* is acceptable, even without new toxicology data (See section 7). In addition, a battery of toxicological studies showed no adverse effects, and the resulting NOAEL was used to determine that the oral exposure via the intended uses is well within a generally acceptable safety margin.

Based on the available data from the literature and generated by DuPont Industrial Biosciences, the company has concluded that α-amylase from *B. licheniformis* (strain JML 1584) is safe and suitable for use in carbohydrate processing, including the manufacture of sweeteners such as high fructose corn syrup (HFCS), and to produce fermentable carbohydrate for yeast fermentation to produce organic acids (e.g., citric and lactic acid) and potable alcohol. The GRAS determination was reviewed by Dr. Michael Pariza, who concurred with DuPont's determination that the enzyme is GRAS for its intended uses, further stating that it is his professional opinion that other qualified experts would also concur in these conclusions.

9. LIST OF APPENDICES

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Appendix 5. *Bacillus licheniformis* safe strain lineage and toxicology summary

Appendix 6. GRAS Concurrence Letter from Dr. Pariza (Redaction in Progress)

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Appendix 1: 21 CFR 170.30

[Code of Federal Regulations]
[Title 21, Volume 3]
[Revised as of April 1, 2005]
From the U.S. Government Printing Office via GPO Access
[CITE: 21CFR170.30]

[Page 13-15]

TITLE 21--FOOD AND DRUGS

CHAPTER I--FOOD AND DRUG ADMINISTRATION, DEPARTMENT OF HEALTH AND HUMAN
SERVICES (CONTINUED)

PART 170_FOOD ADDITIVES--Table of Contents

Subpart B_Food Additive Safety

Sec. 170.30 Eligibility for classification as generally recognized as safe (GRAS).

(a) General recognition of safety may be based only on the views of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. The basis of such views may be either (1) scientific procedures or (2) in the case of a substance used in food prior to January 1, 1958, through experience based on common use in food. General recognition of safety requires common knowledge about the substance throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food.

(b) General recognition of safety based upon scientific procedures shall require the same quantity and quality of scientific evidence as is required to obtain approval of a food additive regulation for the ingredient. General recognition of safety through scientific procedures shall ordinarily be based upon published studies which may be corroborated by unpublished studies and other data and information.

(c)(1) General recognition of safety through experience based on common use in food prior to January 1, 1958, may be determined without the quantity or quality of scientific procedures required for approval of a food additive regulation. General recognition of safety through experience based on common use in food prior to January 1, 1958, shall be based solely on food use of the substance prior to January 1, 1958, and shall ordinarily be based upon generally available data and information. An ingredient not in common use in food prior to January 1,

1958, may achieve general recognition of safety only through scientific procedures.

(2) A substance used in food prior to January 1, 1958, may be generally recognized as safe through experience based on its common use in food when that use occurred exclusively or primarily outside of the United States if the information about the experience establishes that the use of the substance is safe within the meaning of the act (see Sec. 170.3(i)). Common use in food prior to January 1, 1958, that occurred outside of the United States shall be documented by published or other information and shall be corroborated by information from a second, independent source that confirms the history and circumstances of use of the substance. The information used to document and to corroborate the history and circumstances of use of the substance must be generally available; that is, it must be widely available in the country in which the history of use has occurred and readily available to interested qualified experts in this country. Persons claiming GRAS status for a substance based on its common use in food outside of the United States should obtain FDA concurrence that the use of the substance is GRAS.

(d) The food ingredients listed as GRAS in part 182 of this chapter or affirmed as GRAS in part 184 or Sec. 186.1 of this chapter do not include all substances that are generally recognized as safe for their intended use in food. Because of the large number of substances the intended use of which results or may reasonably be expected to result, directly or indirectly, in their becoming a component or otherwise affecting the characteristics of food, it is impracticable to list all such substances that are GRAS. A food ingredient of natural biological origin that has been widely consumed for its nutrient properties in the United States prior to January 1, 1958, without known detrimental effects, which is subject only to conventional processing as practiced prior to January 1, 1958, and for which no known safety hazard exists, will ordinarily be regarded as GRAS without specific inclusion in part 182, part 184 or Sec. 186.1 of this chapter.

(e) Food ingredients were listed as GRAS in part 182 of this chapter during 1958-1962 without a detailed scientific review of all available data and information relating to their safety. Beginning in 1969, the Food and Drug Administration has undertaken a systematic review of the status of all ingredients used in food on the determination that they are GRAS or subject to a prior sanction. All determinations of GRAS status or food additive status or prior sanction status pursuant to this review shall be handled pursuant to Sec. Sec. 170.35, 170.38, and 180.1 of this chapter. Affirmation of GRAS status shall be announced in part 184 or Sec. 186.1 of this chapter.

(f) The status of the following food ingredients will be reviewed and affirmed as GRAS or determined to be a food additive or subject to a prior sanction pursuant to Sec. 170.35, Sec. 170.38, or Sec. 180.1 of this chapter:

(1) Any substance of natural biological origin that has been widely consumed for its nutrient properties in the United States prior to January 1, 1958, without known detrimental effect, for which no health hazard is known, and which has been modified by processes first introduced into commercial use after January 1, 1958, which may reasonably be expected significantly to alter the composition of the substance.

(2) Any substance of natural biological origin that has been widely consumed for its nutrient properties in the United States prior to January 1, 1958, without known detrimental effect, for which no health hazard is known, that has had significant alteration of composition by breeding

or selection after January 1, 1958, where the change may be reasonably expected to alter the nutritive value or the concentration of toxic constituents.

(3) Distillates, isolates, extracts, and concentration of extracts of GRAS substances.

(4) Reaction products of GRAS substances.

(5) Substances not of a natural biological origin, including those for which evidence is offered that they are identical to a GRAS counterpart of natural biological origin.

(6) Substances of natural biological origin intended for consumption for other than their nutrient properties.

(g) A food ingredient that is not GRAS or subject to a prior sanction requires a food additive regulation promulgated under section 409 of the act before it may be directly or indirectly added to food.

(h) A food ingredient that is listed as GRAS in part 182 of this chapter or affirmed as GRAS in part 184 or Sec. 186.1 of this chapter shall be regarded as GRAS only if, in addition to all the requirements in the applicable regulation, it also meets all of the following requirements:

(1) It complies with any applicable food grade specifications of the Food Chemicals Codex, 2d Ed. (1972), or, if specifically indicated in the GRAS affirmation regulation, the Food Chemicals Codex, 3d Ed. (1981), which are incorporated by reference, except that any substance used as a component of articles that contact food and affirmed as GRAS in Sec. 186.1 of this chapter shall comply with the specifications therein, or in the absence of such specifications, shall be of a purity suitable for its intended use. Copies may be obtained from the National Academy Press, 2101 Constitution Ave. NW., Washington, DC 20418, or at the National Archives and Records Administration (NARA). For information on the availability of this material at NARA, call 202-741-6030, or go to: http://www.archives.gov/federal--register/code--of--federal--regulations/ibr--locations.html.

(2) It performs an appropriate function in the food or food-contact article in which it is used.

(3) It is used at a level no higher than necessary to achieve its intended purpose in that food or, if used as a component of a food-contact article, at a level no higher than necessary to achieve its intended purpose in that article.

(i) If a substance is affirmed as GRAS in part 184 or Sec. 186.1 of this chapter with no limitation other than good manufacturing practice, it shall be regarded as GRAS if its conditions of use are not significantly different from those reported in the regulation as the basis on which the GRAS status of the substance was affirmed. If the conditions of use are significantly different, such use of the substance may not be GRAS. In such a case a manufacturer may not rely on the regulation as authorizing the use but must independently establish that the use is GRAS or must use the substance in accordance with a food additive regulation.

(j) If an ingredient is affirmed as GRAS in part 184 or Sec. 186.1 of this chapter with specific limitation(s), it may be used in food only within such limitation(s) (including the category of food(s), the functional use(s) of the ingredient, and the level(s) of use). Any use of such an ingredient not in full compliance with each such established limitation shall require a food additive regulation.

(k) Pursuant to Sec. 170.35, a food ingredient may be affirmed as GRAS in part 184 or Sec. 186.1 of this chapter for a specific use(s) without a general evaluation of use of the ingredient. In addition to the use(s) specified in the regulation, other uses of such an ingredient may also be GRAS. Any affirmation of GRAS status for a specific use(s), without a general evaluation of use of the ingredient, is subject to reconsideration upon such evaluation.

(l) New information may at any time require reconsideration of the GRAS status of a food ingredient. Any change in part 182, part 184, or Sec. 186.1 of this chapter shall be accomplished pursuant to Sec. 170.38.

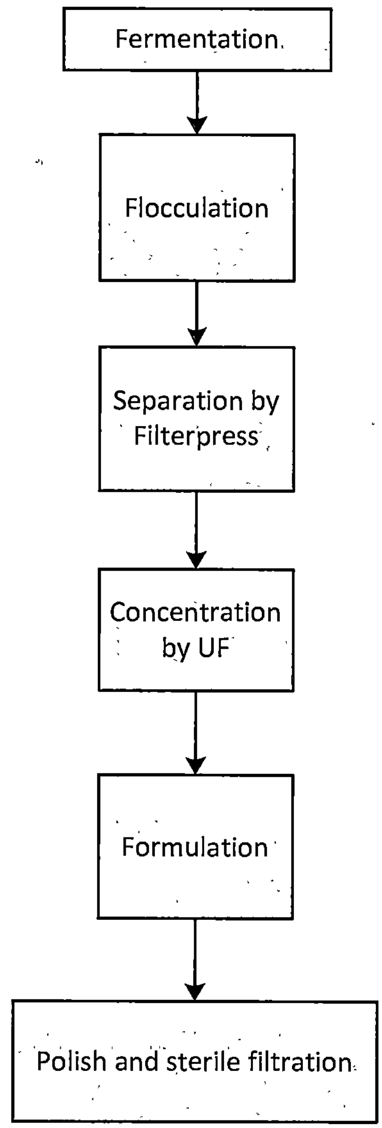
[42 FR 14483, Mar. 15, 1977, as amended at 49 FR 5610, Feb. 14, 1984; 53 FR 16546, May 10, 1988]

Appendix 2 Amino Acid sequence of *B. licheniformis* C16F α-amylase

AATNGTMMQYFEWYVPNDGQQWNRLRTDAPYLSSVGITAVWTPPAYKGTSQADVGYGPYDLYDLGEF
NQKGTVRTKYGTKGELKSAVNTLHSNGIQVYGDVVMNHKAGADYTENVTAVEVNPSNRYQETSGEYNIQ
AWTGFNFPGRGTTYSNWKWQWFHFDGTDWDQSRSLSRIFKFHGKAWDWEVSSENGNYDYLMYADYD
YDHPDVVNEMKKWGVWYANEVGLDGYRLDAVKHIKFQFLKDWVDNARAATGKEMFTVGEYWQNDLGA
LNNYLAKVNYNQSLFDAPLHYNFYAASTGGGYYDMRNILNNTLVASNPTKAVTLVENHDTQPGQSLESTV
QPWFKPLAYAFILTRSGGYPSVFYGDMYGTKGTTTREIPALKSKIEPLLKARKDYAYGTQRDYIDNPDVIG
WTREGDSTKAKSGLATVITDGPGGSKRMYVGTSNAGEIWYDLTGNRTDKITIGSDGYATFPVNGGSVSV
WVQQ

Appendix 3: *B. licheniformis* C16F α-amylase production processes

Ultra-Filtered Concentrate (UFC)



Appendix 4: Certificates of Analysis

Ultra-Filtered Concentrate (UFC) of C16F (aka Level 10) Amylase

DuPont Industrial Biosciences

1700 Lexington Avenue
Rochester, New York 14606

CERTIFICATE OF ANALYSIS

PRODUCT: Level 10 Amylase clarified concentrate

LOT NUMBER: 20138069

ASSAY	UNIT	FOUND
ENZYME ACTIVITY		
Amylase	DLU/g	59314
MICROBIOLOGICAL ANALYSIS		
Total Viable Count	CFU/ml	<1
Total Coliforms	CFU/ml	<1
E. coli	/25ml	Negative by test
Salmonella	/25ml	Negative by test
Staphylococcus aureus	/ml	Negative by test
Anaerobic Sulfite Reducing Bacteria	CFU/ml	Negative by test
Production Strain	/ml	Negative by test
Antibacterial Activity	/ml	Negative by test
PHYSICAL PROPERTIES		
pH		6.4
Specific Gravity		1.02
Percent Solids	%w/w	4.31
OTHER ASSAYS		
Heavy Metals, as Pb	mg/kg	<30
Lead	mg/kg	<5
Cadmium	mg/kg	<0.5
Mercury	mg/kg	<0.5
Arsenic	mg/kg	<3

19-Sep-2013
Date

Kelly A. Altman
QA/QC Department

This certificate of analysis was electronically generated and therefore has not been signed.

DuPont Industrial Biosciences

1700 Lexington Avenue
Rochester, New York 14606

CERTIFICATE OF ANALYSIS

PRODUCT: Level 10 Amylase clarified concentrate

LOT NUMBER: 20138088

ASSAY	UNIT	FOUND
ENZYME ACTIVITY		
Amylase	DLU/g	66309
MICROBIOLOGICAL ANALYSIS		
Total Viable Count	CFU/ml	<1
Total Coliforms	CFU/ml	<1
E. coli	/25ml	Negative by test
Salmonella	/25ml	Negative by test
Staphylococcus aureus	/ml	Negative by test
Anaerobic Sulfite Reducing Bacteria	CFU/ml	Negative by test
Production Strain	/ml	Negative by test
Antibacterial Activity	/ml	Negative by test
PHYSICAL PROPERTIES		
pH		6.3
Specific Gravity		1.03
Percent Solids	%w/w	7.12
OTHER ASSAYS		
Heavy Metals, as Pb	mg/kg	<30
Lead	mg/kg	<5
Cadmium	mg/kg	<0.5
Mercury	mg/kg	<0.5
Arsenic	mg/kg	<3

19-Sep-2013
Date

Kelly A. Altman
QA/QC Department

This certificate of analysis was electronically generated and therefore has not been signed.

DuPont Industrial Biosciences

1700 Lexington Avenue
Rochester, New York 14606

CERTIFICATE OF ANALYSIS

PRODUCT: Level 10 Amylase clarified concentrate

LOT NUMBER: 20138109

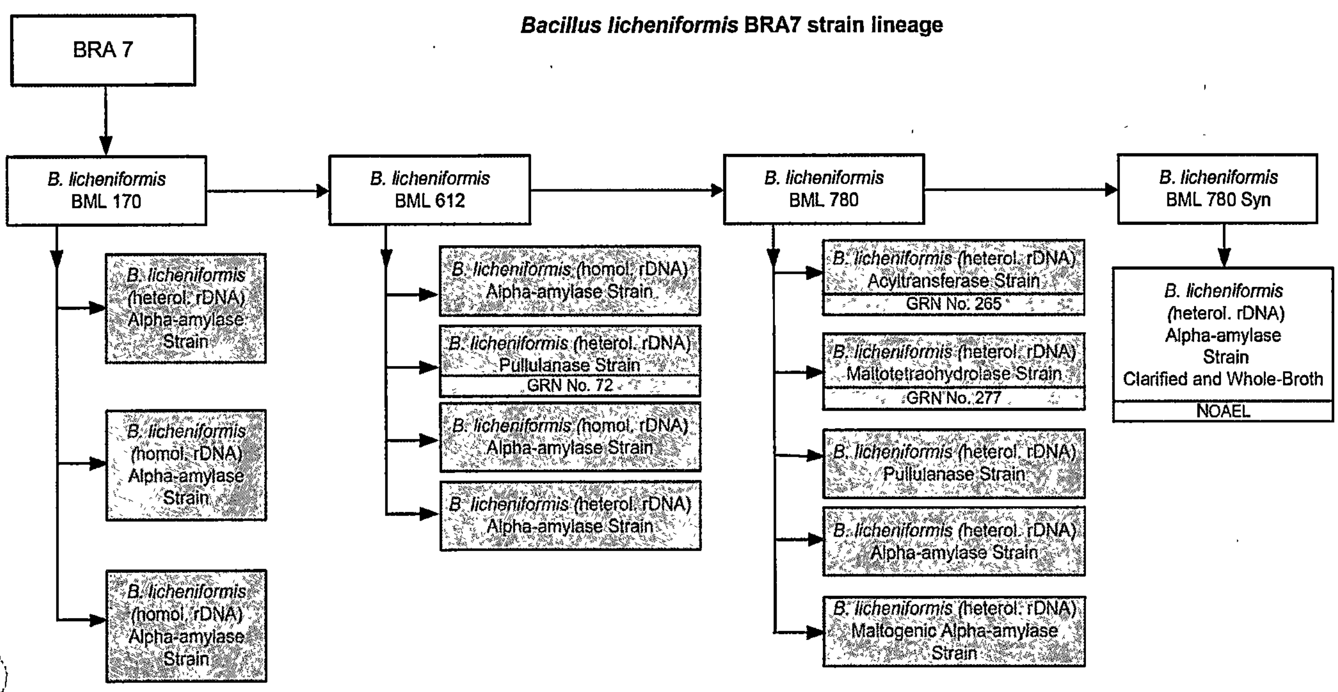
ASSAY	UNIT	FOUND
ENZYME ACTIVITY		
Amylase	DLU/g	61670
MICROBIOLOGICAL ANALYSIS		
Total Viable Count	CFU/ml	<1
Total Coliforms	CFU/ml	<1
E. coli	/25ml	Negative by test
Salmonella	/25ml	Negative by test
Staphylococcus aureus	/ml	Negative by test
Anaerobic Sulfite Reducing Bacteria	CFU/ml	Negative by test
Production Strain	/ml	Negative by test
Antibacterial Activity	/ml	Negative by test
PHYSICAL PROPERTIES		
pH		5.9
Specific Gravity		1.03
Percent Solids	%w/w	5.38
OTHER ASSAYS		
Heavy Metals, as Pb	mg/kg	<30
Lead	mg/kg	<5
Cadmium	mg/kg	<0.5
Mercury	mg/kg	<0.5
Arsenic	mg/kg	<3

19-Sep-2013
Date

Kelly A. Altman
QA/QC Department

This certificate of analysis was electronically generated and therefore has not been signed.

Appendix 5: *Bacillus licheniformis* safe strain lineage and toxicology summary



All commercial enzymes derived from this Safe Strain Lineage were determined to be GRAS, with GRAS Notices submitted for review by the US FDA for enzymes from strains designated with green horizontal banners (indicating the GRAS Notice number).

The subject strain is the **Alpha-amylase** producing strain highlighted in yellow.
The safety of the **Alpha-amylase** enzyme is supported by repeated testing of other enzymes produced by members of this Safe Strain Lineage. The orange-colored boxes indicate strains for which we toxicology tests were conducted.

The NOAELs for these **Alpha-amylase** preparations are used to calculate the safety margins in the respective intended uses.

A Determination of Safe Strain Lineage for *Bacillus licheniformis* host strain BRA7

The species *Bacillus licheniformis* has been used as a production organism for enzymes by DuPont Industrial Biosciences (legacy Genencor), since 1989.

Genencor has conducted numerous toxicology and genotoxicity studies with enzyme preparations derived from various *Bacillus licheniformis* strains derived from *Bacillus licheniformis* host strain BRA7. An evaluation and summary of the data are discussed in this memorandum. All toxicology studies sponsored by Genencor strictly follow corresponding OECD guidelines and are conducted in compliance with all current Good Laboratory Practice Standards. A summary table of the toxicology studies can be found in Figure 1.

All the enzymes discussed below have been evaluated by GRAS panels who have determined that the enzymes are safe for their intended uses and are Generally Recognized As Safe (GRAS).

A. Enzymes derived from *Bacillus licheniformis* BML 170

A.1. Alpha-amylase from *Bacillus licheniformis* (heterol. rDNA) strain

A battery of genotoxicity assays was conducted and under the conditions of these assays, the AA enzyme was not a mutagen in a bacterial reverse mutation assay (Ames assay) and was not a clastogen or an aneugen in an *in vitro* chromosomal aberration assay with human peripheral lymphocytes in the presence and absence of metabolic activation. The potential of the enzyme to induce systemic toxicity was investigated after repeated daily oral administration of the ultra-filtered concentrate of the product in Wistar rats of both sexes. The enzyme was given by gavage for 28 consecutive days at 0, 20, 100 or 500 mg/kg body. Under the conditions of this study, the NOAEL (no observed adverse effect level) was established at the highest dose tested, 500 mg /kg bw/day.

A.2. Alpha-amylase from *Bacillus licheniformis* (homol. rDNA) strain

A battery of genotoxicity assays was conducted and under the conditions of these assays, the AA enzyme was not a mutagen in a bacterial reverse mutation assay (Ames assay) and was not a clastogen or an aneugen in an *in vitro* chromosomal aberration assay with human peripheral lymphocytes in the presence and absence of metabolic activation. The systemic toxicity potential of the enzyme has not been investigated, but was not expected to be different from the AA enzyme in A.1 above.

A.3. Alpha-amylase from *Bacillus licheniformis* (homol. rDNA) strain

This enzyme is a low pH α-amylase produced by a variant of an alpha-amylase (homol. rDNA) strain. The genotoxicity potential of the enzyme was investigated in a bacterial reverse mutation assay (Ames assay) and a chromosomal aberration assay with human lymphocytes. The enzyme was not a mutagen or clastogen in both the presence and absence of metabolic activity. The potential toxicity after oral administration (gavage) was investigated in the rat for 13 consecutive weeks. Groups of animals received 0, 625, 1250 or 2,500 mg/kg/day of the ultra-filtered concentrate corresponding to 29.25, 58.50 and 117 mg TOS/kg/day. No treatment related adverse effects were noted in this study and the NOAEL was established at the highest dose tested, 2,500 mg/kg/day or 117 mg TOS/kg/day.

References

Bio-Research Laboratories, Inc.: 13-week gavage subchronic toxicity study. Final report No. 87629, December 10, 1996.

Microbiological Associates, Inc.: *In vitro* chromosomal aberrations. Final report NO. G96B072.346, February 20, 1997.

Microbiological Associates, Inc.: Bacterial reverse mutation assay. Final report NO. G96B072.502, December 13, 1996.

B. Products derived from *Bacillus licheniformis* BML 612

B.1. Alpha-amylase from *Bacillus licheniformis* (homol. rDNA) strain

This enzyme is a low pH α amylase produced from a *Bacillus licheniformis* (homol. rDNA) strain. The mutagenic potential of the enzyme was investigated in a bacterial reverse mutation assay (Ames assay) and an *in vitro* chromosomal aberration assay with human peripheral lymphocytes. Under the conditions of these assays, the enzyme was not a mutagen or clastogen in both the presence and absence of metabolic activation. The systemic toxicity potential was investigated in male and female rats treated with the enzyme for 13 consecutive weeks. The ultra-filtered concentrate was given by oral gavage to groups of rats at 0, 625, 1,250 or 2,500 mg/kg/day. There were no treatment related effects. The NOAEL was established at the highest dose tested, 2,500 mg/kg/day.

References

Harlan Laboratories: Acute oral toxicity in the rat – Fixed dose method. Report No. 2420/0016, June 01, 2009.

Harlan Laboratories: Acute inhalation toxicity (nose only) in the rat. Report No. 2420/0017, July 15, 2009.

Harlan Laboratories: Acute dermal irritation in the rabbit. Report No. 2420/0018, June 01, 2009.

Harlan Laboratories: Acute eye irritation in the rabbit. Report No. 2420/0019, June 01, 2009.

Harlan Laboratories: Local lymph node assay in the mouse. Report No. 2420/0020, August 05, 2009.

Harlan Laboratories: *Salmonella typhimurium* and *Escherischia coli* reverse mutation assay. Report No. 2420/0021, May 15, 2009.

Harlan Laboratories: Chromosome aberration test in human lymphocytes *in vitro*. Report No. 2420/0022, August 7, 2009.

Harlan Laboratories: 90-day repeated oral (gavage) toxicity study in the rat. Report No. 2420/0023, October 5, 2009

B.2. Pullulanase from *Bacillus licheniformis* (heterol. rDNA) strain

This enzyme is a pullulanase enzyme produced by a *Bacillus licheniformis* (homol. rDNA) strain with applications in foods and its safety has been investigated. Pullulanase was not an irritant to the eyes and skin. Pullulanase was practically non-toxic based on acute inhalation and acute ingestion studies. In genotoxicity studies, Pullulanase was not a mutagen in a bacterial reverse mutation assay (Ames assay) and was not a clastogenic or an aneugen in an *in vitro* chromosomal aberration assay with human peripheral lymphocytes in both the presence and absence of metabolic activation. Daily oral (gavage)

administration of ultra-filtered concentrate of Pullulanase for 90 consecutive days up to and including a dose level of 2,500 mg/kg did not result in any treatment-related adverse effects in rats. A NOAEL (no observed adverse effect level) was established at 2,500 mg/kg/day of the UF concentrate. Based on a specific gravity of 1.034, a total protein of 69.79 mg/ml and a total organic solid content of 9.82%, this NOAEL (2,500 mg/kg/day) corresponds to 168.9 mg total protein/kg/day or 237.64 mg TOS/kg/day.

References

BioReliance No. AA16GE.507.BTL, Bacterial reverse mutation assay with an independent repeat assay, August 1999.

BioReliance No. AA16GE.341.BTL, *In vitro* mammalian chromosome aberration test, September 1999.

ClinTrials BioResearch No. 88873, A 13-week oral gavage toxicity study of Pullulanase in the albino rats, August 1999.

IRDC No. 713-002, 4-week dietary toxicity study in rats with Pullulanase, June 1994.

IRDC No. 713-003, Primary dermal irritation test in rabbits with Pullulanase, February 1994.

IRDC No. 713-004, Primary eye irritation study in rabbits with Pullulanase, February 1994.

IRDC No. 713-005, Acute inhalation toxicity evaluation in rats with Pullulanase, April 1994.

IRDC No. 713-006, Bacterial reverse mutation assay (Ames assay) with Pullulanase, Feb 1994 (Genesys Final Report No. 93027, February 1994).

IRDC No. 713-007, *In vitro* forward mutation assay using the L5178Y/tk+/- mouse lymphoma cells with Pullulanase, Feb 1994 (Genesys Final Report No. 93028, February 1994).

IRDC No. 713-009, *In vivo* mouse bone marrow chromosome aberration test with Pullulanase, August 1994 (Genesys Final report No. 93030, August 1994).

B.3 Alpha-amylase from *Bacillus licheniformis* (homol. rDNA) strain

The safety of the α-amylase enzyme produced from a *Bacillus licheniformis* (homol. rDNA) strain was assessed in a battery of toxicology studies investigating its acute oral, inhalation, irritation, skin sensitization, mutagenic and systemic toxicity potential. The enzyme was not an eye or skin irritant and was not acutely toxic by ingestion. It is not a dermal sensitizer based on the results of the local lymph node assay. A battery of genotoxicity assays was conducted and under the conditions of these assays and was determined not to be a mutagen in the bacterial reverse mutation assay (Ames assay) and was not a clastogen or an aneugen in the *in vitro* chromosomal aberration assay with human peripheral lymphocytes in both the presence and absence of metabolic activation. Daily administration of the enzyme's ultra-filtered concentrate by gavage for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL was established at the highest dose tested, 80 mg total protein/kg bw/day corresponding to 110 mg TOS/kg bw/day.

References

Covance Laboratories: 13-week gavage sub-chronic toxicity study with alpha amylase. Final report No. 7043-100, December 7, 1999.

MA BioServices Inc.: *In vitro* mammalian chromosome aberration test with alpha amylase. Final report No. G98AG08.341, June 12, 1998.

MA BioServices Inc.: Bacterial reverse mutation assay with alpha amylase. Final report NO. G98AG08.507, August 27, 1998.

B.4 Alpha-amylase from *Bacillus licheniformis* (heterol. rDNA) strain

The AA enzyme was not mutagenic in the Ames assay and was not clastogenic in the mammalian system (*in vitro* chromosomal aberration assay with human peripheral lymphocytes) in both the presence and absence of metabolic activation. The systemic toxicity after repeated daily oral administration (gavage) of the ultra-filtered concentrate was investigated in Sprague Dawley rats of both sexes for 90 consecutive days at 0, 16, 32, or 64 mg total protein/kg body weight. These doses corresponded to 0, 175, 350 or 700 mg TOS/kg bw/day, respectively. There were no treatment-related effects in this study. Under the conditions of this assay, the NOAEL (no observed adverse effect level) was established at the highest dose tested, 64 mg total protein/kg bw/day or 700 mg TOS/kg bw/day.

References

Scantox Study No. 57860, Acute dermal irritation study in the rabbit with Alpha Amylase, April 20, 2005.
Scantox Study No. 57861, Ocular irritation test in the rabbit with Alpha Amylase, March 8, 2005.
Scantox Study No. 57831, Ames Test with Alpha Amylase, April 14, 2005.
Scantox Study No. 57832, *In vitro* mammalian chromosome aberration test performed with human lymphocytes, Alpha Amylase, August 15 2005.
Scantox Study No. 58136, A 13-week oral (gavage) toxicity study in rats with Alpha Amylase, June 24, 2005.

C. Products derived from *Bacillus licheniformis* BML 780

C.1. Acyltransferase from *Bacillus licheniformis* (heterol. rDNA) strain

Acyltransferase's safety was assessed in a battery of toxicology studies. The enzyme was not an irritant to the eyes and skin and was practically non-toxic based on an acute oral ingestion study. In genotoxicity studies, the enzyme was not mutagenic in the bacterial reverse mutation assay (Ames assay), was not clastogenic or aneugenic in the *in vitro* chromosomal aberration assay with human peripheral lymphocytes, and was not aneugenic in an *in vivo* mouse micronucleus assay in both the presence and absence of metabolic activation. The potential systemic toxicity of the enzyme after repeated daily oral administration of the ultra-filtered concentrate was investigated in SPF Sprague Dawley rats for 90 consecutive days. Groups of rats of both sexes were gavaged daily with 0, 4.56, 13.68 or 41.00 mg total protein/kg body weight corresponding to 0, 13.0, 39.0 and 116.9 mg TOS/kg bw/day, respectively. Daily oral administration of the enzyme up to and including a dose level of 41 mg total protein/kg bw/day did not result in any manifestation of adverse health effects. A NOAEL was established at 41 mg total protein or 116.9 mg TOS/kg bw/day.

References

Scantox Study No. 62125, Acute dermal irritation study in the rabbit with Acyltransferase, September 2006.
Scantox Study No. 62124, Acute eye irritation/corrosion study in the rabbit with Acyltransferase, September 2006.
Scantox Study No. 62123, Acute oral toxicity study in the rat with Acyltransferase. September 2006.
Scantox Study No. 62127, Acyltransferase, Ames Test, October 2006.
Scantox Study No. 62126, *In vitro* mammalian chromosome aberration test performed with human lymphocytes, Acyltransferase, 2006.
Scantox Study No. 64415, Mouse micronucleus test with Acyltransferase, November 2006.

Scantox Study No. 62129, A13-week oral (gavage) toxicity study in rats with Acyltransferase, October 2006.

C.3. Maltotetraohydrolase from *Bacillus licheniformis* (heterol. rDNA) strain

The safety of the maltotetraohydrolase produced by a *Bacillus licheniformis* (heterol. rDNA) strain that was assessed in a battery of toxicology studies investigating its acute oral, irritation, mutagenic and systemic toxicity potential. The enzyme was not a skin irritant, was not acutely toxic by ingestion and is a mild eye irritant. A battery of genotoxicity assays was conducted and under the conditions of these assays, the enzyme was not a mutagen in a bacterial reverse mutation assay (Ames assay) and was not a clastogen or an aneugen in an *in vitro* chromosomal aberration assay with human peripheral lymphocytes in both the presence and absence of metabolic activation. The potential of the maltotetraohydrolase amylase to induce systemic toxicity after repeated daily oral (gavage) administration was investigated in Wistar rats of both sexes. Ultra-filtered enzyme concentrate was given for 90 consecutive days by gavage at 0, 23.7, 47.4 or 79 mg total protein/kg body weight corresponding to 0, 27.3, 54.5 or 90.9 mg TOS/kg bw/day, respectively. Under the conditions of this assay, the NOAEL (no observed adverse effect level) was established at the highest dose tested, 79 mg total protein/kg bw/day corresponding to 90.0 mg TOS/kg bw/day.

References

SafePharm Lab Study No. 2420/0005, Acute dermal irritation study in the rabbit with maltotetraohydrolase, 15 April 2008.
SafePharm Lab Study No. 2420/0004, Acute eye irritation/corrosion study in the rabbit with maltotetraohydrolase, 28 April 2008.
SafePharm Lab Study No. 2420/0003, Acute oral toxicity study in the rat with maltotetraohydrolase, fixed dosed method, 13 May 2008.
SafePharm Lab Study No. 2420/0006, Reverse mutation assay – Ames Test with maltotetraohydrolase, 12 June 2008.
SafePharm Lab Study No. 2420/0007, Chromosome aberration test in human *lymphocytes in vitro* with maltotetraohydrolase, 06 June 2008.
SafePharm Lab Study No. 2420/0008, 90 day repeated oral (gavage) toxicity study in the rat with maltotetraohydrolase, 14 October 2008.

C.4. Pullulanase from *Bacillus licheniformis* (heterol. rDNA) strain

The safety of Truncated PU is assessed in a battery of toxicology studies investigating its genotoxic and systemic toxicity potential. Under the conditions of the mutagenicity assays Truncated PU is not a mutagen or clastogen. Daily administration of Truncated PU by gavage for 90 continuous days did not result in overt signs of systemic toxicity or adverse effects on clinical chemistry, hematology, functional observation tests and macroscopic and histopathologic examinations. Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established at the highest dose tested, 500 mg TOS/kg bw/day corresponding to 260 mg TP/kg bw/day.

References

BioReliance: H-30648: Bacterial reverse mutation assay; Report No. AD69TA.507001.BTL; Dupont No. 20265-513; Final report dated July 22, 2013.

BioReliance: H-30648: *In vitro* mammalian chromosome aberration test in human peripheral blood lymphocytes; Report No. AD69TA.341.BTL; Dupont No. 20265-544; Final report dated July 30, 2013.

Dupont Haskell Global Centers: H-30648 Subchronic toxicity 90 day gavage study in rats; Report No. 20265-1026; Final report dated February 6, 2014.

C.2. Alpha-amylase from *Bacillus licheniformis* (heterol. rDNA) strain

The safety of the AA enzyme was assessed in a battery of toxicology studies investigating its irritation, acute oral, genotoxic and systemic toxicity potential. The enzyme was not an eye or skin irritant. Genotoxicity assays were conducted and under the conditions of these assays, the enzyme was not a mutagen in a bacterial reverse mutation assay (Ames assay) and was not a clastogen or an aneugen in an *in vitro* chromosomal aberration assay with human peripheral lymphocytes in both the presence and absence of metabolic activation. The systemic toxicity was investigated in SPF Sprague Dawley rats. Ultra-filtered concentrate was given by gavage daily for 90 consecutive days at 0, 4.96, 12.4 and 37.2 mg total protein/kg bw corresponding to 0, 8.9, 22.27 and 66.81 mg TOS/kg bw/day, respectively. Daily administration of GC 358 by gavage for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL was established at 37.2 mg total protein/kg bw/day corresponding to 66.81 mg TOS/kg bw/day.

References

Harlan Laboratories No. 41100560: Alpha-amylase, Acute dermal irritation in the rabbit, June 10, 2011.

Harlan Laboratories No. 41100561: Alpha-amylase, Acute eye irritation in the rabbit, July 14, 2011.

Harlan Laboratories No. 41100559: Alpha-amylase, Acute oral toxicity in the rat – Fixed dose method, July 18, 2011.

Harlan Laboratories No. 41100562: Alpha-amylase, Reverse mutation assay "Ames Test" using *Salmonella typhimurium* and *Escherichia coli*, September 7, 2011.

Harlan Laboratories No. 41100563: Alpha-amylase, Chromosome aberration test in human lymphocytes *in vitro*, September 16, 2011.

Harlan Laboratories No. 41100564: Ninety day repeated dose oral (gavage) toxicity study in the rat – Alpha-amylase, December 6, 2011.

C.5. Maltogenic Alpha-amylase from *Bacillus licheniformis* (heterol. rDNA) strain

The safety of the maltogenic alpha-amylase was assessed in a battery of toxicology studies investigating its dermal and eye irritation, acute oral, genotoxic and systemic toxicity potential. Maltogenic alpha-amylase was not an eye or skin irritant. Genotoxicity assays were conducted and under the conditions of these assays Maltogenic alpha-amylase was not a mutagen in a bacterial reverse mutation assay (Ames assay) and was not a clastogen or an aneugen in an in vitro chromosomal aberration assay with human peripheral lymphocytes in both the presence and absence of metabolic activation. The systemic toxicity of Maltogenic alpha-amylase was investigated in Wistar rats. Ultra-filtered concentrate of Maltogenic alpha-amylase was given by gavage daily for 90 consecutive days at 0, 13.9, 27.8, and 55.6 mg total

protein/kg bw corresponding to 0, 20, 40, and 80 mg TOS/kg bw/day, respectively. Daily administration of Maltogenic alpha-amylase by gavage for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL was established at 55.6 mg total protein/kg bw/day corresponding to 80 mg TOS/kg bw/day.

References

BioReliance: H-30648: Bacterial reverse mutation assay; Report No. AD69TA.507001.BTL; Dupont No. 20265-513; Final report dated July 22, 2013.

BioReliance: H-30648: *In vitro* mammalian chromosome aberration test in human peripheral blood lymphocytes; Report No. AD69TA.341.BTL; Dupont No. 20265-544; Final report dated July 30, 2013.

Dupont Haskell Global Centers: H-30648 (Truncated PU) Subchronic toxicity 90-day gavage study in rats; Report No. 20265-1026; Final report dated February 6, 2014.

D. Products derived from *Bacillus licheniformis* BML 780-syn

D.1. Alpha-amylase from *Bacillus licheniformis* (heterol. rDNA) strain

The safety of Alpha amylase (C16F UFC) is assessed in a battery of toxicology studies investigating its genotoxic and systemic toxicity potential. Under the conditions of the mutagenicity assays Alpha amylase (C16F UFC) is not a mutagen or clastogen. Daily administration of Alpha amylase UFC by gavage for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at 500 mg TOS/kg bw/day (corresponding to 272 mg TP/kg bw/day).

References

BioReliance: H-30929: Bacterial reverse mutation assay; Report No. AD84GP.507001.BTL; Dupont No. 20558-513; Final report dated February 04, 2014.

Dupont Haskell Global Centers: H-30929: *In vitro* mammalian chromosome aberration test in human peripheral blood lymphocytes; Report No. 20558-544; Final report dated February 21, 2014.

MPI Research: H-30929: Subchronic toxicity 90 day oral gavage study in rats; Report No. 125-180; Final report dated October 2014.

SUMMARY

<u>Acute toxicity and Irritation Studies</u>

All enzyme preparations produced from various strains of *Bacillus licheniformis* are practically non-toxic by ingestion (oral LD_{50} greater than 2000 mg/kg) and are not irritating to the skin or eyes.

<u>Genotoxicity</u>

Numerous genotoxicity studies were conducted and all enzyme preparations produced from various strains of *Bacillus licheniformis* are not mutagenic, not aneugenic and not clastogenic.

<u>Systemic Toxicity</u>

A review of all repeated oral administration studies in rodents suggests that no specific target organ toxicity can be identified with enzyme preparations produced from various strains of *Bacillus licheniformis*. There were no adverse effects on body weight, feed and water consumption and daily clinical observations. There were no effects on ophthalmologic examination, hematology, clinical chemistry, urinalysis and functional observation battery. At necropsy, there was no specific target organ toxicity that can be attributed to these enzyme preparations.

DISCUSSION

The safety of enzyme preparations produced from various strains of *Bacillus licheniformis* was investigated for their potential irritation, genotoxicity and systemic toxicity in studies designed following OECD guidelines. Studies investigating the systemic toxicity of enzymes from *Bacillus licheniformis* were designed to follow the OECD Guideline No. 408 (Sub-chronic oral toxicity – Rodent: 90 day study) (adopted 21 September 1998) and the EPA Guideline OPPTS 870.3100 (August 1998). Studies investigating the genotoxic potential were designed to follow the OECD Guideline No. 471 (Bacterial reverse mutation assay) (May 30, 2008) and Guideline No. 473 (Chromosome Aberration Assay) (May 30, 2008). OECD Guideline No. 429 (Skin sensitization: Local lymph node assay) (April 24, 2002) was used to detect the potential for skin sensitization. All studies sponsored by DuPont Industrial Biosciences (legacy Genencor) were performed in compliance with all current Good Laboratory Practice Standards.

A review of all toxicology studies conducted with enzyme preparations produced by different strains of *Bacillus licheniformis* indicates that, regardless of the production organism strain, all enzyme preparations are not irritating to the skin and eyes, are not skin sensitizers, are not mutagenic, clastogenic or aneugenic in genotoxicity assays and do not adversely affect any specific target organ. The NOAEL obtained from the oral (gavage) administration studies was always the highest dose tested. Thus, the existing data substantiate and demonstrate that the *Bacillus licheniformis* host strain BRA7 lineage is indeed a safe strain lineage and all enzyme preparations produced by these *Bacillus licheniformis* strain are safe and suitable for their intended uses. Due to the consistency of the findings from enzyme preparations derived from different *Bacillus licheniformis* host strain BRA7 derived strains, it is expected that any new enzyme preparation produced using the *Bacillus licheniformis* host strain BRA7 lineage would behave similarly from a toxicological standpoint. Therefore, it can be concluded that Genencor can utilize this *Bacillus licheniformis* host strain BRA7 safe strain lineage to produce other enzymes without conducting new toxicology and/or safety studies to demonstrate their safety.

TABLE 1

SUMMARY OF TOXICOLOGY DATA FROM ENZYME PREPARATIONS PRODUCED FROM DIFFERENT *BACILLUS LICHENIFORMIS* STRAINS

***BACILLUS LICHENIFORMIS* host strain BRA7 STRAIN LINEAGE**													
	HOST STRAIN BML 170			**HOST STRAIN BML 612**				**HOST STRAIN BML 780**					**HOST STRAIN BML 780-Syn**
Enzyme	Alpha-amylase	Alpha-amylase	Alpha-amylase	Alpha-amylase	Pullulanase	Alpha-amylase	Alpha-amylase	Acyl Transferase	Alpha-amylase	Maltotetrao-hydrolase	Maltogenic Alpha-amylase	Pullulanase	Alpha-amylase
Genotoxicity	No effects	No effects	No effects	No effects	No effects	No effects	No effects	No effects	No effects	No effects	No effects	No effects	No effects
Systemic Toxicity	None	No Data	None	None	None	None	None	None	None	None	None	None	None
NOAEL (TOS/kg/day)		No Data	117 mg	420.75 mg	237.64 mg	700 mg	110 mg	116.9 mg	90 mg	66.81 mg	80 mg	500 mg	500 mg
NOAEL (total protein/kg/d)				280.75 mg	168.9 mg	64 mg	80 mg	41 mg	79 mg	37.2 mg	55.6 mg	260 mg	272 mg
NOAEL (UFC/kg/d)	500 mg/kg/d (28-day oral)		2500 mg/kg/d	2500 mg/kg/d									

Appendix 6: GRAS Concurrence letter from Dr. Pariza

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(608) 271-5169
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Michael W. Pariza, Member

April 10, 2016

Vincent Sewalt, PhD
Senior Director, Product Stewardship & Regulatory
DuPont Industrial Biosciences
Danisco US, Inc.
925 Page Mill Road
Palo Alto, CA 94304

RE: GRAS opinion on the intended uses in food processing of Danisco/DuPont's C16F AA α-amylase produced by *Bacillus licheniformis* JML 1584 (GICC03437)

Dear Dr. Sewalt,

I have reviewed the information that you provided on Danisco/DuPont's C16F AA α-amylase, which is produced by *Bacillus licheniformis* JML 1584 (GICC03437). C16F AA α-amylase is a synthetic variant of the native *Cytophaga sp.* α-amylase, and will be used in carbohydrate processing including the manufacture of sweeteners such as high fructose corn syrup (HFCS), fermentation to produce organic acids (i.e. lactic and citric), and the production of potable alcohol.

In evaluating the C16F AA α-amylase product, I considered the biology of *B. licheniformis* and *Cytophaga sp.*; information that you provided on the C16F AA gene and α-amylase protein structure including its similarity to other α-amylases that have histories of safe use in food manufacture; the construction of *B. licheniformis* JML 1584 (GICC03437); and other pertinent information that is available in the peer-reviewed scientific literature.

Bacillus licheniformis is a common soil microorganism that has not been associated with pathogenicity or toxigenicity for humans, other animals, or plants. This species is listed in the Food Chemicals Codex as a source of carbohydrase and protease enzyme preparations used in food processing. The FDA has affirmed that a mixed carbohydrase and protease enzyme product derived from *B. licheniformis* is GRAS for use in the production of certain foods (21 CFR 184.1027). GRP 5G0415 (converted to GRN 000072) cites published reports on the cloning and expression of proteins in *B. licheniformis* for use in food products, and FDA issued a 'no objection' letter for the uses of *B. deramificans* pullulanase expressed by *B. licheniformis* GICC03088 as described in GRN00072.

Danisco/DuPont has developed a lineage of safe enzyme production strains from *B. licheniformis* Bra7, a classical industrial strain developed from its wild-type parent via classical strain improvement methodologies. *Bacillus licheniformis* GICC03088, described in GRN 000072, is a member of this safe strain lineage of *B. licheniformis* Bra7 enzyme production strains. *Bacillus licheniformis* Bra7 and strains derived from it have been used to produce α-amylase since 1989 with food grade versions in use for grain processing since 1998.

Cytophaga sp. belong to a large and diverse bacterial group, referred to as the *Cytophaga- Flavobacteria* cluster. They are described as unicellular, gliding, nonspore-forming Gram-negative rods, and are found in soil and aquatic (particularly marine) environments. *Cytophaga sp.* are especially proficient in degrading various biopolymers such as cellulose, chitin, and pectin. The physiology and genetics of the *Cytophaga* sp. are poorly understood, but some members of the genus are reported to be fish pathogens (Carson *et al., Journal of Fish Diseases* 16:209-218, 1993). Pathogenicity is a complex process that typically involves the expression of specialized invasive elements called virulence factors, none of which are associated with α-amylase or its gene.

The C16F AA α-amylase gene, which was introduced into *B. licheniformis* Bra7 to construct *B. licheniformis* JML 1584 (GICC03437), is a synthetic, engineered variant of the native α-amylase gene found in a *Cytophaga sp.* that was originally isolated from a corn field in Taiwan (Chiou *et al.,* Current Microbiol. 56:597-602, 2008.) In this application, the native *Cytophaga sp.* α-amylase gene was used as a starting template for the *in vitro* synthesis of the C16F AA α-amylase gene, which has improved catalytic performance over the native enzyme. The fully characterized synthetic gene was then introduced into *B. licheniformis* Bra7 to generate *B. licheniformis* JML 1584 (GICC03437), using cloning reagents and methodologies that are accepted by the scientific community for the construction of organisms that produce food-grade ingredients.

The native α-amylase enzyme of the donor *Cytophaga sp.* isolate is a typical Glycoside Hydrolase family 13, subfamily 5 (GH13-5) α-amylase, based on amino acid sequence homologies. GH13-5 α-amylases , which have been widely studied, are found mostly in *Bacillus* species. Comparison of amino acid sequences revealed 75% homology between C16F AA α-amylase and *B. amyloliquefaciens* α-amylase. *Bacillus amyloliquefaciens* α-amylase has a history of use in food (21CFR184.1148) and has been studied extensively with regard to improving its commercial applications via protein engineering (Lee *et al.,* J.

Biochem. 139: 1007-1015, 2006). FDA has issued 'no objection' letters for the use in food of a 'hybrid' *B.amyloliquefaciens/B. licheniformis* α-amylase (GRN 000022) and a protein engineered 'hybrid' *B.amyloliquefaciens/B. licheniformis* α-amylase (GRN 000079).

Given these considerations, I concur with your conclusion that C16F AA α-amylase is *substantially equivalent* to the *B. amyloliquefaciens* α-amylase and its derivatives, which are the subjects of GRNs 000022 and 000079.

Analysis of the C16F AA α-amylase amino acid sequence revealed no evidence that the C16F AA α-amylase might be allergenic via oral exposure. A BLAST search for homology of the mature C16F AA protein sequence with known toxins or antinutrients was also performed, and revealed no evidence that the C16F AA α-amylase might be toxigenic.

The safety of C16F AA α-amylase intended for use in food applications was investigated using a battery of toxicology studies that included determining the potential for genotoxic and systemic toxicity. Based on a 90-day subchronic studies in Charles River CD rats, the NOAEL was determined to be the highest dose tested, 272 mg Total Protein (equal to 500 mg TOS/kg bw/day). This corresponded to a safety margin of 245.5 for C16F AA α-amylase in all food applications, which is well above the 100-fold safety factor that is generally accepted for food ingredients and food processing aids.

Based on the foregoing, I concur with your conclusion, that *Bacillus licheniformis* JML 1584 (GICC03437) is safe to use for the production of C16F AA α-amylase, to be used in carbohydrate processing including the manufacture of sweeteners such as high fructose corn syrup (HFCS), fermentation to produce organic acids (i.e. lactic and citric), and the production of potable alcohol.

I also concur with your conclusion that the C16F AA α-amylase preparation, produced by *Bacillus licheniformis* JML 1584 (GICC03437) in a manner that is consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food-grade specifications, is *substantially equivalent* to the *B. amyloliquefaciens* α-amylase and its derivatives, which are the subjects of GRNs 000022 and 000079, and GRAS (Generally Recognized As Safe) for use in carbohydrate processing including the manufacture of sweeteners such as high fructose corn syrup (HFCS), fermentation to produce organic acids (i.e. lactic and citric), and the production of potable alcohol.

Finally, I concur with your conclusion that the C16F AA α-amylase preparation, produced by *Bacillus licheniformis* JML 1584 (GICC03437) in a manner that is consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food-grade specifications, is *Generally Recognized As Safe* (GRAS) for use in carbohydrate processing including the manufacture of sweeteners such as high fructose corn syrup (HFCS), fermentation to produce organic acids (i.e. lactic and citric), and the production of potable alcohol.

It is my professional opinion that other qualified experts would also concur in these conclusions.

Please note that this is a professional opinion directed at safety considerations only and not an endorsement, warranty, or recommendation regarding the possible use of the subject product by you or others.

Sincerely,

(b) (6)

Michael W. Pariza, Ph.D.
Member, Michael W. Pariza Consulting, LLC
Professor Emeritus, Food Science
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SUBMISSION END