ORIGINAL SUBMISSION

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July 17, 2015

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 beta-glucanase enzyme preparation produced by *Bacillus subtilis* expressing the gene encoding beta-glucanase from *B. subtilis* 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.
925 Page Mill Road
Palo Alto, CA 94304

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

Beta-glucanase enzyme preparation from *Bacillus subtilis* expressing the gene encoding the BglS beta-glucanase from *B. subtilis.*

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

The beta-glucanase is used as processing aid in brewing and potable alcohol production.

**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**

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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)

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)

# A Beta-Glucanase Enzyme

# Preparation Derived from

# *Bacillus subtilis*

# Expressing the Beta-Glucanase Gene

# From

# *Bacillus subtilis*

# Is Generally Recognized As Safe

# For Use in Food Processing

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

**July 17, 2015**

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# TABLE OF CONTENTS

## 1. GENERAL INTRODUCTION

The beta-glucanase enzyme preparation under consideration is produced by submerged fermentation of *Bacillus subtilis* overexpressing the native *BglS* gene from *B. subtilis* encoding the wild-type beta-glucanase enzyme (hereafter named Beta-glucanase BglS).

The enzyme product is intended for use in brewing and potable alcohol production. In these applications, the beta-glucanase BglS will primarily be replacing beta-glucanase from one of the other available commercial sources. In all of these applications, beta-glucanase will be used as a processing aid, where the enzyme is either not present in the final food or present in insignificant quantities as inactive residue, having no function or technical effect in the final food.

Other beta-glucanases currently in use include beta-glucanases and cellulases from other microorganisms, most notably *Bacillus amyloliquefaciens, Aspergillus niger, Trichoderma reesei, Talaromyces emersoni and Humicola insolens* The beta-glucanase from *B. subtilis* has been in commerce as a minor component of other *B. subtilis* enzyme preparation, as BglS beta-glucanase is present as side activity in earlier *B. subtilis* production organisms. Only relatively recently however, enzyme preparations with commercially viable levels of beta-glucanase production were introduced. DuPont Industrial Biosciences first determined beta-glucanase from *B. subtilis* to be GRAS in 2012.

The accepted name of the principle enzyme activity is endo-1,3 (4)-beta-glucanase. Other names used are beta-glucanase, endo-1,3-β-D-glucanase; laminarinase; laminaranase; β-1,3-glucanase; β-1,3-1,4-glucanase; endo-1,3-β-glucanase; endo-β-1,3(4)-glucanase; endo-β-1,3-1,4-glucanase; endo-β-(1→3)-D-glucanase; endo-1,3-1,4-β-D-glucanase; endo-β-(1-3)-D-glucanase; endo-β-1,3-glucanase IV; endo-1,3-β-D-glucanase; 1,3-(1,3;1,4)-β-D-glucan 3(4)-glucanohydrolase.

The enzyme catalyzes endohydrolysis of (1->3)- or (1->4)-linkages in beta-D-glucans when the glucose residue whose reducing group is involved in the linkage to be hydrolyzed is itself substituted at C-3.

The EC number of the enzyme is 3.2.1.6 and the CAS number is 62213-14-3

The information provided in the following sections is the basis of our determination of GRAS status of this beta-glucanase BglS enzyme preparation.

Our safety evaluation in Section 7 includes an evaluation of the production strain, the enzyme and the manufacturing process, as well as a determination of dietary exposure to the preparation.

The safety of the production organism must be the prime consideration in assessing the safety of an enzyme preparation intended for food use (Pariza & Johnson, 2001; Pariza & Foster, 1983). The safety of the production organism (*B. subtilis*) for the beta-glucanase BglS is discussed in Sections 2 and 7. Another essential aspect of the safety evaluation of enzymes derived from

genetically modified microorganisms is the identification and characterization of the inserted genetic material (Pariza & Johnson, 2001; Pariza & Foster, 1983; IFBC, 1990; EU SCF, 1991; OECD, 1993; Berkowitz and Maryanski, 1989). The genetic modifications used to construct this production organism are well defined and are described in Section 2. The safety evaluation described in Section 7 shows no evidence to indicate that any of the cloned DNA sequences and incorporated DNA code for or express a harmful toxic substance.

### 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 beta-glucanase enzyme preparation produced by *Bacillus subtilis* expressing the gene encoding beta-glucanase from *B. subtilis* 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 beta-glucanase enzyme preparation from *Bacillus subtilis* expressing the gene encoding the beta-glucanase from *B. subtilis* (beta-glucanase BglS).

### 1.4 Applicable Conditions of Use

The beta-glucanase is used as a processing aid in brewing and potable alcohol production.

### 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 organism is a strain of *B. subtilis* (strain CF 624B-1), which has been genetically modified to over express a gene for the production of the *B. subtilis* BglS beta-glucanase. *Bacillus subtilis* has already been used for decades for the production of food enzymes with no known reports of adverse effects to human health or the environment (de Boer and Diderichsen, 1991). The US Food and Drug Administration reviewed the safe use of food-processing enzymes from well-characterized recombinant microorganisms, including *B. subtilis* (Olempska-Beer *et al.* 2006). An extensive risk assessment and human risk assessment of *B. subtilis*, including its history of commercial use has been published by the US Environmental Protection Agency (1997). It was concluded that *B. subtilis* 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). One expression cassette was used to introduce the *B. subtilis* gene encoding beta-glucanase BglS under the regulation of the endogenous *aprE* promoter and *Bacillus amyloliquefaciens apr* as the terminator. The expression cassette was integrated into the recipient chromosome.

### 2.2 Host Micoorganism

The host microorganism *B. subtilis* BG125 is a laboratory strain, previously described by Dedonder *et al.*, (1977), which was obtained as *B. subtilis* strain 1A10 from the Bacillus Genetic Stock Center, Ohio State University, Columbus, Ohio. This strain was developed into a host strain by Genencor (a division of Danisco), now known as DuPont Industrial Biosciences. It is derived from the well-nown *B. subtilis* type strain 168 via classical genetics as described in Dedonder *et al.*, (1977).

Several genomic genes have been deleted from the host strain as well, resulting in the final parent strain BG3934.

An intermediate strain in this construction, *B. subtilis* BG3594-3 was recognized by the Dutch authorities as Risk Class 1.

### 2.3 Donor Microorganism

The donor strain is the same as the host organism.

### 2.4 Beta-glucanase BglS Expression Cassettes

The genetic modification of the *B. subtilis* host involved recombinant DNA techniques to introduce multiple copies of the endogenous gene encoding the wild type *B. subtilis* BglS beta-glucanase into the chromosome of the *B. subtilis* host.

The modification employed a method by which an expression cassette, consisting of the *aprE* promoter, beta-glucanase BglS gene and *Bacillus amyloliquefaciens apr* terminator and the chloramphenicol resistance marker gene from plasmid pC194 (originally isolated from *S. aureus* but widely recognized to be naturally present in *Bacillus*), is introduced into the host genome, at the site of the endogenous alkaline protease *aprE* gene, without any vector sequences remaining in the final production strain.

The genetic construction was evaluated at every step to assess the incorporation of the desired functional genetic information and the final construct was verified by Southern blot analysis to confirm the copy number of the integrated beta-glucanase cassettes and the absence of bacterial vector DNA.

### 2.5 Stability of the Introduced Genetic Sequences

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

### 2.6 Antibiotic Resistance Gene

No new antibiotic resistance gene was introduced into the production microorganism, but rather the endogenous chloramphenicol resistance marker was employed to select the production strain.

### 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 | Endo-1,3(4)-beta-glucanase |
| IUB Number: | 3.2.1.6 |
| CAS Number: | 62213-14-3 |

| | |
|---|---|
| Reaction catalyzed: | Endohydrolysis of (1->3)- or (1->4)-linkages in beta-D-glucans when the glucose residue whose reducing group is involved in the linkage to be hydrolyzed is itself substituted at C-3 |
| Other names: | endo-1,3 (4)-beta-glucanase. Other names used are beta-glucanase, endo-1,3-β-D-glucanase; laminarinase; laminaranase; β-1,3-glucanase; β-1,3-1,4-glucanase; endo-1,3-β-glucanase; endo-β-1,3(4)-glucanase; endo-β-1,3-1,4-glucanase; endo-β-(1→3)-D-glucanase; endo-1,3-1,4-β-D-glucanase; endo-β-(1-3)-D-glucanase; endo-β-1,3-glucanase IV; endo-1,3-β-D-glucanase; 1,3-(1,3;1,4)-β-D-glucan 3(4)-glucanohydrolase |

### 3.2 Amino Acid Sequence

The amino acid sequence of *Bacillus subtilis* Beta-glucanase BglS enzyme is shown in Appendix 2. The nucleotide sequence is available in GenBank under accession number X00754. The sequence of *Bacillus subtilis* Beta-glucanase BglS is similar to various other beta-glucanases isolated from commercially relevant bacterium, e.g., it is 100% homologous with the wild type *Bacillus subtilis* beta-glucanase and 94% homologous with *Bacillus amyloliquefaciens* beta-glucanase which were both recognized as GRAS in 21CFR184.1148.

## 4. MANUFACTURING PROCESS

This section describes the manufacturing process for the beta-glucanase enzyme which follows standard industry practice (Kroschwits, 1994; Aunstrup K *et al.*, 1979; unstrup 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 also 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 beta-glucanase concentrate are standard ingredients used in the enzyme industry practice (Kroschwits, 1994; Aunstrup *et al.*, 1979; Aunstrup, 1979). All the raw materials conform to the specifications of the Food Chemicals Codex (FCC), 9th edition, 2014 (US Pharmacopeia, 2014), except for those raw materials that do not appear in the FCC. For those not appearing in the FCC, internal requirements have been made 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.

The antifoam used in the fermentation and recovery is used in accordance with the cGMP per the FDA correspondence to Enzyme Technical Association submission acknowledging the listed antifoams dated September 11, 2003. The maximum use level of the antifoam in the production process is $\leq$0.15%.

Glucose (which may be produced from wheat) and soy flour are used in the fermentation process, but both will be consumed by the microorganism as nutrients. No other major allergen substances are used in the fermentation, recovery processes or in the formulation.

**4.2 Fermentation Process**

The beta-glucanase enzyme is manufactured by submerged fermentation of a pure culture of the genetically modified strain of *B. subtilis* 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. subtilis* 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 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 ultrafiltered concentrate is stabilized by final formulation to contain ~40% glycerol, sodium citrate, citric acid, and small amounts of sodium benzoate and potassium sorbate, at pH 4.8. The remaining is water.

## 5. COMPOSITION AND SPECIFICATIONS

### 5.1 Quantitative Composition

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: | 40,500 BBU/g |
| Glycerol: | 40.0% |
| Sodium Citrate ($Na_3C_6H_5O_7x2H_2O$): | 1.4% |
| Citric Acid ($H_3C_6H_5O_7$): | 0.6% |
| Sodium benzoate: | 0.2% |
| Potassium sorbate: | 0.1% |
| Remainder is water (with minor % of fermentation components) | |
| pH | 4.8 |

### 5.2 Specifications

Beta-glucanase BglS meets the purity specifications for enzyme preparations set forth in the FCC 9th edition (2014). 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 lot of product used in toxicological testing is given in Appendix 4, verifying that it meets FCC (U.S. Pharmacopeia, 2012) and JECFA (2006) specifications for enzyme preparations.

## 6. APPLICATION

### 6.1 Mode of Action

Beta-glucanase functions in the endohydrolysis of (1→3)- or (1→4)-linkages in β-D-glucans when the glucose residue whose reducing group is involved in the linkage to be hydrolyzed is itself substituted at C-3.

### 6.2 Uses and Use Level

Beta-glucanase BglS is used as a processing aid in brewing and potable alcohol production. Cereal grains used in these applications such as barley, rye, and wheat contain high levels of beta-glucan that give high viscosity due to water-binding capacity. High viscosity has negative effects in brewing and ethanol production because it limits solid concentration in mashing and reduces efficiency. The beta-glucanase enzyme product is used to break down beta-glucan resulting in reduced viscosity of the slurry which in turn helps support the mixing, separation and filtration process.

#### 6.2.1 Uses

*Brewing*

In brewing, the BglS beta-glucanase enzyme is added in mashing, where it hydrolyzes the beta-glucans of the mash (mixture of milled gelatinized malt, gelatinized adjunct, and water) reducing wort viscosity. It will be used in the mashing of malted cereal, unmalted cereal and other plant sources including barley, corn, wheat, rye, milo, rice, tapioca and potatoes.

After mashing, the wort is separated from the spent grains via filtration and ultimately boiled for 1-1.5 hrs for sterilization. With a temperature of 100 °C during this process the enzyme product is completely inactivated. The resultant process liquors (worts) are fermented, typically by yeast, to produce ethanol.

*Potable alcohol*

The BglS beta-glucanase is used to hydrolyze the beta-glucans in the mashing step to reduce wort viscosity. It will be used in the mashing of malted cereal, unmalted cereal and other plant sources including barley, corn, wheat, rye, milo, rice, tapioca and potatoes.

In potable alcohol applications, solids are separated from the fermentation slurry at the end of fermentation and any enzyme protein precipitate is removed with the solids. The liquids are then distilled with the temperature at the bottom of the still at approximately 82 °C. The distilled

alcohol is subsequently filtered through a molecular sieve (Madson *et al.,* 2003) at temperatures well over boiling (149 °C) to adsorb further traces of water (and along with it, any water soluble protein). Therefore, the enzyme product is not present/active in the end product.

6.2.2 Use Levels

For brewing and potable alcohol, 1.25 mg BglS protein will be used per kg grist, which corresponds to 36.56 mg TOS/kg grist.

### 6.3 Enzyme Residues in the Final Foods

DuPont Industrial Biosciences expects beta-glucanase to be inactivated or removed during the subsequent production processes for all applications.

In brewing, the BglS beta-glucanase enzyme is added in mashing, where it hydrolyzes the beta-glucans of the mash (mixture of milled gelatinized malt, gelatinized adjunct, and water) reducing wort viscosity. It will be used in the mashing of malted cereal, unmalted cereal and other plant sources including barley, corn, wheat, rye, milo, rice, tapioca and potatoes. After mashing, the wort is separated from the spent grains via filtration and ultimately boiled for 1-1.5 hrs for sterilization. With a temperature of 100 °C during this process the enzyme product is completely inactivated. The resultant process liquors (worts) are fermented, typically by yeast, to produce ethanol.

In potable alcohol applications, solids are separated from the fermentation slurry at the end of fermentation and any BglS beta-glucanase enzyme protein precipitate is removed with the solids. The liquids are then distilled with the temperature at the bottom of the still at approximately 82 °C. The distilled alcohol is subsequently filtered through a molecular sieve (Madson *et al.,* 2003) at temperatures well over boiling (149 °C) to adsorb further traces of water (and along with it, any water soluble protein). Not only is the BglS beta-glucanase protein irreversibly denatured at this temperature, given the poor solubility of enzyme protein in alcohol virtually none of it (<1%) will be carried in the evaporated alcohol.

## 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 subtilis* strains used in enzyme manufacture meet these criteria for non-toxigenicity and non-pathogenicity.

### 7.1.1 Safety of the host

*B. subtilis* 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. subtilis* is considered to be a benign organism that does not possess traits that cause disease. This also applies to the DuPont Industrial Biosciences (legacy Danisco) *B. subtilis* host strain, which has been demonstrated to be non-pathogenic, non-toxigenic and not cytotoxic.

The potential risk associated with the use of this bacterium in fermentation facilities is low (US EPA, 1997).

Recently scientists with the US Food and Drug Administration reviewed the safe use of food-processing enzymes from recombinant microorganisms, including *B. subtilis* (Olempska-Beer *et al.*, 2006). An extensive risk assessment of *B. subtilis*, including its history of commercial use has been published by the US EPA (1997). It was concluded that *B. subtilis* strains used for enzyme manufacture are neither pathogenic nor toxigenic to humans. It is, however, prudent to ascertain the safety of the production strain as certain food-borne illness related strains may produce surfactin, a membrane spanning lipopeptide and amylolysin, a heat-stable toxin regarded to be a virulence factor (Apetroaie-Constantin *et al.*, 2009; Logan, 2012).

Despite the documented safety of *Bacillus,* strains derived from the *B. subtilis* safe strain lineage and comparable to the current production strain were tested for pathogenicity and toxicity by DuPont Industrial Biosciences (see Appendix 5). The conclusion of the research was that no toxic substances were produced by the strain, i.e. that it is non-pathogenic and non-toxigenic.

The production organism of the beta-glucanase enzyme preparation, the subject of this submission is a strain of *B. subtilis*, CF 624B-1, which has been genetically modified to over express a gene for the production of the *B. subtilis* BglS beta-glucanase.

All the *B. subtilis* are derived from the *Bacillus subtilis subsp. subtilis* strain 168 (Burkholder and Giles, 1947), the basic laboratory strain which has been completely sequenced (Kunst *et al.*, 1997). The parent strain BG125 derived from strain 168 carries three auxotrophic mutations: *trpC2*, *hisA* and *thr5,* and was described as QB917 (Dedonder *et al.*, 1977). This strain can be obtained from the Bacillus Genetic Stock Center, Ohio State University, Columbus, Ohio, as strain 1A10.

Beta-glucanase BglS produced by this specific production strain has been determined to be non-toxicogenic through the following toxicology tests: 1) acute dermal irritation study in rabbits, 2) acute eye irritation/corrosion study in rabbits 3) bacterial reverse mutation – Ames assay, 4) *In vitro* mammalian chromosomal aberration test performed with human lymphocytes, and 5) 13-week oral (gavage) toxicity study in rats. In addition, all of the food/feed grade products produced by the lineage to which this strain pertains were determined to be safe for their intended uses, and are the subject of numerous GRAS determinations.

From the information reviewed, it is concluded that the production organism *B. subtilis* strain CF 624B-1 provides no specific risks to human health and is safe to use as the production organism of Beta-glucanase BglS. The strain is non-pathogenic and non-toxigenic.

7.1.2 Safety of the Donor Organism

In this case, the donor organism is the same as the host, *B. subtilis*, therefore the safety assessment is the same.

## 7.2 Safety of the Manufacturing Process

The manufacturing process for the production of Beta-glucanase BglS is 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. The process, described in Appendix 3 is conducted in accordance with food Good Manufacturing Practice (GMP) as set forth in 21 CFR Part 110. The resultant product meets the general requirements for enzyme preparations of the FCC, 9th edition (US Pharmacopeia, 2014) and JECFA (2006) enzyme specifications.

## 7.3 Safety of *Bacillus subtilis* Beta-glucanase

7.3.1 Allergenicity

According to Pariza and Foster (1983), there have been no confirmed reports of allergies in consumers caused by enzymes used in food processing. Beta-glucanase has been used in food processes for many years and has generated no known safety concerns.

In 1998 the Association of Manufacturers of Fermentation Enzyme Products (AMFEP) Working Group on Consumer Allergy Risk from Enzyme Residues in Food reported on an in-depth analysis of the allergenicity of enzyme products. They concluded that there are no scientific indications that small amounts of enzymes in bread and other foods can sensitize or induce allergy reactions in consumers, and that enzyme residue in bread and other foods do not represent any unacceptable risk to consumers. Further, in a recent investigation of possible oral

allergenicity of 19 commercial enzymes used in the food industry, there were no findings of clinical relevance even in individuals with inhalation allergies to the same enzymes, and the authors concluded "that ingestion of food enzymes in general is not considered to be a concern with regard to food allergy (Bindslev-Jensen *et al.*, 2006).

Despite this lack of general concern, the potential that Beta-glucanase BglS could be a food allergen was assessed by comparison with sequences of known allergens. Based on the sequence homology alone, it was concluded that the *B. subtilis* Beta-glucanase BglS is unlikely to pose a risk of food 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."

The *B. subtilis* Beta-glucanase BglS expressed in *B. subtilis* is given below in FASTA format, without its secretion signal.

QTGGSFFDPFNGYNSGFWQKADGYSNGNMFNCTWRANNVSMTSLGEMRLALTSPAYNKFD
CGENRSVQTYGYGLYEVRMKPAKNTGIVSSFFTYTGPTDGTPWDEIDIEFLGKDTTKVQFNYY
TNGAGNHEKIVDLGFDAANAYHTYAFDWQPNSIKWYVDGQLKHTATNQIPTTPGKIMMNLWN
GTGVDEWLGSYNGVNPLYAHYDWVRYTKK

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 1897 (version released Jan 12, 2015) peer-reviewed allergen sequences (listed in http://www.allergenonline.org/databasebrowse.shtml) revealed multiple stretches throughout the peptide sequence with over 35% identity to to Asp f 9 or Probable glycosidase crf 1 from

*Aspergillus fumingatus*. The maximum sequence identity to Asp f 9 is 40.7% (NCBI gi|2879890|emb|CAA11266.1|rAsp f 9, partial).

FASTA alignments of the above sequence with known allergens revealed match also to the Asp f 9 (using E-value <0.1 as the cut-off) with 28.9 % identity and $4.4x10^{-9}$ as the E-score[1] using the full sequence search capabilities. Asp f 9 is not identified as food allergen by the World Health organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee (http://www.allergen.org/viewallergen.php?aid=125).

Although cautioned against in Codex (2009), researched by Herman *et al.* (2009) and further elaborated by Ladics *et al.* (2011) and on AllergenOnline.org that there is no evidence that a short contiguous 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 precautionary search. Performing this search produced no sequence matches with known allergens.

Microbial enzymes acting environmental allergens have yet to be conclusively demonstrated to be active via the oral route. This concept was evaluated extensively in a recently published study (Bindslev-Jensen *et al.*, 2006) that failed to indicate positive reactions to 19 orally challenged commercial enzymes in a double blind placebo controlled food challenge study with subjects with positive skin prick tests for the same allergens. The authors concluded that positive skin prick test results are of no clinical relevance to food allergenicity, and that ingestion of food enzymes in general is not a concern with regard to food allergy.

In conclusion, based on the sequence homology alone, *B. subtilis* Beta-glucanase BglS is unlikely to pose a risk of food allergenicity.

As for all enzyme products, an MSDS for the Beta-glucanase BglS product would include a precautionary statement that inhalation of enzyme mist/dust may cause allergic respiratory reactions, including asthma, in susceptible individuals on repeated exposure.

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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.

7.3.2 Safety of use in food

In addition to the allergenicity assessment described above, the safety of this beta-glucanase has also been established using the Pariza and Johnson (2001) decision tree, see Appendix 6. Although the Pariza and Johnson evaluation resulted in the conclusion to accept the enzyme preparation as safe without toxicology testing, the safety of the 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.

7.3.3 Safety Assessment

A review of all toxicology studies conducted with enzyme preparations produced by different strains of the DuPont Industrial Biosciences *B. subtilis* safe strain lineage 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. Due to the consistency of the findings from enzyme preparations derived from different *B. subtilis* strains, it is expected that any new enzyme preparation produced from *B. subtilis* strains would have a similar toxicological profile. (Appendix 5)

In addition, DuPont Industrial Biosciences has conducted five studies on Beta-glucanase BglS enzyme produced from *B. subtilis* CF 624B-1. The results are evaluated, interpreted and assessed in this document. The test material, an ultra-filtrate concentrate (UFC) used in all toxicology investigations, has the following characteristics (see also Appendix 4):

| | |
|---|---|
| Lot No.: | 20128099 |
| Physical: | Fermentation liquid, brown |
| Enzyme activity: | 160365 BBU/g |
| pH: | 6.57 |
| Specific gravity: | 1.03 g/ml |
| Total protein: | 102.29 mg/ml |
| TOS: | 10.16 % |

The studies include:

A. Acute dermal irritation study in rabbits

B. Acute eye irritation/corrosion study in rabbits

C. Bacterial reverse mutation assay – Ames assay

D. *In vitro* mammalian chromosomal aberration test performed with human lymphocytes

E. 13-week oral (gavage) toxicity study in CD rats

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.

Summaries are included below.

**1) Acute dermal irritation study in rabbits (sequential approach)**

a. Procedure:

The objective of this study was to assess the local irritant effect of Beta-glucanase BglS. This study was conducted according to the method recommended in the OECD Guideline No. 404, April 2002. This study was conducted in a stepwise manner. Initially, 5/10 of a milliliter of the test substance was applied to the skin of one rabbit for 4 hours. Since no dermal irritation was noted in this rabbit, a confirmatory test was completed with two additional healthy rabbits.

Approximately 24 hours prior to application, all animals were prepared by clipping the dorsal area and the trunk. Five tenth of one milliliter was applied to one 6 $cm^2$ intact dose site and covered with 1 inch x 1 inch, 4-ply gauze pad. The pad and entire trunk of each animal were then wrapped with semi-occlusive tape. Elizabethan collars were placed on each rabbit. After 4 hours of exposure, the pads and collars were removed and the test sites were cleansed of any residual test substance. The grade of skin reaction was scored according to the Draize's scoring system immediately following patch removal and at 30-60 minutes, 24, 48 and 72 hours after patch removal.

b. Results

No deaths or overt signs of toxicity were observed in this study. No effects on feed consumption and weight gain were recorded. No dermal irritation was observed at any dose site during the study.

c. Evaluation

According to the provisions of Directive 67/548/EEC amended by Commission Directive 2001/59/EC of 6 August 2001, Annex VI, classification is not required.

According to the United Nations Globally Harmonized System of Classification and Labeling of Chemicals (GHS), under the conditions of this study, classification is not required.

### 2) Acute Eye Irritation/Corrosion Study in the Rabbit

a. Procedure

The objective of this study was to assess the ocular irritation potential of Beta-glucanase BglS. This study was conducted according to the method recommended in the OECD Guideline No. 405, 24 April 2002 and evaluated according to the Commission Directive 2001/59/EC of 6 August 2001.

The study was conducted in a stepwise fashion. Initially, 1/10 of a milliliter of the test substance was instilled into the conjunctival sac of the right eye of one rabbit. The left eye remained untreated and served as control. The grade of ocular reaction was recorded at 1, 24, 48 and 72 hours post instillation and evaluated by the method of Draize *et al.* At the 24-hour reading, fluorescein was instilled and then rinsed with 0.9% NaCl. The eye was then examined with an UV-light to detect corneal damage. Since there was no significant ocular irritation noted in this rabbit, the test was repeated on two additional rabbits by the same procedure.

b. Results

Conjunctival redness (score = 1) was noted in the treated eye of one rabbit at the 1-hour period with clearing by 24 hours. No ocular irritation was noted in the remaining two rabbits.

c. Evaluation

According to the provisions of Directive 67/548/EEC amended by Commission Directive 2001/59/EC of 6 August 2001, Annex VI, classification is not required.

According to the United Nations Globally Harmonized System of Classification and Labeling of Chemicals (GHS), under the conditions of this study, classification is not required.

### 3) Bacterial Reverse Mutation Assay – Ames assay.

a. Procedure:

The objective of this assay was to assess the potential of Beta-glucanase BglS 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 *Escherischia 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.

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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.

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

b. Results:

In the screening assay, Beta-glucanase BglS was not toxic to the 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 of S-9mix or with tester strains TA1535, TA1537 and WP2 *uvr*A in the absence of S-9 mix. 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 first confirmatory assay, six dose levels (15, 50, 150, 500, 1500, and 5000 µg TP/plate) were tested. However, contamination was observed on the vehicle control and test article treated-plates; therefore, both assays were retested.

In the retested confirmatory assay, six dose levels (15, 50, 150, 500, 1500, and 5000 µg TP/plate) were tested. Neither precipitate nor toxicity was observed. 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, Beta-glucanase BglS has not shown any evidence of mutagenic activity in the Ames assay in both presence and absence of metabolic activation.

**4) *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes.**

a. Procedure

The objective of this assay was to investigate the potential of Beta-glucanase BglS 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.

Beta-glucanase BglS 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. Ten concentrations of Beta-glucanase BglS were used in the preliminary assay and at least 4 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 preliminary assay, all cultures with or without S-9 mix were treated for 4 hours and continuously for 20 hours in the absence of S-9 mix. In the definitive assay, cultures with and without S-9 mix were exposed to the test article for 4 hours, and continuously for 20 hours in the absence of S-9 mix. For the preliminary and the definitive assays, cells were collected 20 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.

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

b. Results

In the preliminary assay, the dose levels ranged from 0.5 to 5000 µg TP/ml. Exposure period was 4 hours for both cultures with and without S9 mix, and continuously for 20 hours in the absence

of S-9 mix. All cells were harvested after 20 hours after treatment initiation. No visible precipitation of the test material in the culture medium was observed. Substantial toxicity (at least 50% reduction in mitotic index relative to the vehicle control) was observed at dose levels ≥ 5000 µg/ml in the non-activated 4-hour groups, at dose levels ≥ 1500 µg/ml in the activated 4-hour groups, and at dose levels ≥ 500 µg/ml in the non-activated 20-hour groups.
Based on those findings, dose levels ranging from 25 to 5000 µg/ml were used in the definitive assays.

In the definitive assays, substantial toxicity (at least 50% reduction in mitotic index relative to the vehicle control) was not observed in the non-activated 4 hours exposure groups. Substantial toxicity was observed in the activated 4 hours exposure groups at dose levels ≥ 4000 µg TP/ml. Substantial toxicity was observed in the non-activated 20 hours exposure groups at dose levels ≥ 250 µg TP/ml. Based on these findings, the doses chosen for microscopic analysis were:

Non-activated 4 hours exposure groups: 1000, 2500 and 5000 µg TP/ml
Activated 4 hours exposure groups: 750, 1500 and 4000 µg TP/ml
Non-activated 20 hours exposure groups: 100, 150 and 250 µg TP/ml

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, Beta-glucanase BglS 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 non-cytotoxic concentration as 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.

**5) 13-week Oral (Gavage) Toxicity Study in CD Rats.**

a. Procedure

The objective of this study was to investigate the potential of Beta-glucanase BglS 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 (0.9% saline), 100, 300 or 1000 mg TOS/kg bw/day. The dosing volume was 5 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.

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

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 Beta-glucanase BglS by oral gavage to CD rats at doses of 0, 100, 300 or 1000 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, 1000 mg TOS/kg bw/day.

**CONCLUSION**

The safety of Beta-glucanase BglS produced by *B. subtilis* was assessed in a battery of toxicology studies investigating its irritation, genotoxic and systemic toxicity potential. The Beta-glucanase BglS is not an eye and skin irritant, not a mutagen or clastogen.

Daily administration of Beta-glucanase BglS by gavage for 13 continuous weeks in rats did not result in overt signs of systemic toxicity. A NOAEL was established at 1000 mg TOS/kg bw/day equivalent to 977.5 mg total protein/kg bw/day.

## 7.4 Overall Safety Assessment and Human Exposure

### 7.4.1 Identification of the NOAEL

In the 90-day oral (gavage) study in rats, a NOAEL was established at 1000 mg TOS/kg bw/day (equivalent to 977 mg total protein/kg bw/day or 589.5 GAU/kg bw/day). The study was designed based on OECD guideline No. 408 and conducted in compliance with both the FDA Good Laboratory Practice Regulations and the OECD Good Laboratory Practice. Since human exposure to beta-glucanase is through oral ingestion, selection of this NOAEL is thus appropriate.

NOAEL = 1000 mg TOS/kg bw/day
= 977 mg Total Protein/kg bw/day

7.4.2. Human Exposure to Beta-glucanase BglS

To estimate worst-case exposure to Beta-glucanase BlgS, the relatively conservative Budget method was used. According to the Budget method (Douglass et al., 1997), the solid food consumption is assumed to be 100 kcal/kg and 50 kcal/kg for children and adults, respectively. Using 60 kg as a default body weight for adults, the maximum lifetime energy intake of 50 kcal/kg bw/day for solid foods corresponds to 25 g/kg bw/day.

The intake of beverage according to the Budget method is 100 ml/kg bw/day (non-milk beverage) assuming that 25% of beverage have been treated with enzyme. The daily consumption according to the Budget method is then 25 ml/kg bw.

Estimated intake of BglS beta-glucanase

BglS beta-glucanase will be used as a processing aid where the enzyme is not present in the final food or present as inactive residue in negligible amounts.

Estimation of daily consumption of Beta-glucanase BglS from its potential uses in major commodities is based on the Budget method as proposed by EFSA (2011):

- *Brewing*

Beta-glucanase BglS is used in the brewing industry at 3,518 BBU/kg grist which corresponds to 36.56 mg TOS/kg grist. It is assumed that 17 kg grist gives 100 L beer and that 100% Beta-glucanase BglS remains in the beer after processing. The maximum concentration of Beta-glucanase BglS found in beer:

| | | |
|---|---|---|
| Max application level grist | 1.25 | mg active enzyme protein/kg grist |
| Max application level grist | 3517.5 | BBU/kg grist |
| Max application level grist | 36.56 | mg TOS/kg grist |
| Yield of beer from grist | 5.88 | L/kg grist |
| Enzyme activity in beer | 6.22 | mg TOS/L beer |

- *Potable alcohol production*

Since the application rate of Beta-glucanase BglS in brewing is the same as that of potable alcohol but the exposure is higher (due to lack of the distillation process), the higher exposure from brewing will be used in this risk assessment to represent a worst case scenario.

- Taking the respective process yield into account, the resulting worst-case exposure in beer is higher (on an equal alcohol content basis) than the theoretical exposure via potable alcohol. It is reasonable to equalize intake based on % alcohol, as 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.

- Moreover, 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.

Hence, the higher exposure from brewing was used in our risk assessment to represent a worst case scenario exposure via intake of liquids regardless of whether this is from consumption from beer, or distilled spirits, with the assumption that 25% of all consumed beverages are manufactured from raw materials treated with the beta-glucanase.

Total Maximum Daily Intake calculations

- ***Solid food contribution***

Since Beta-glucanase BglS is only used in brewing and potable alcohol applications there is no contribution to the dietary exposure from solid foods.

- ***Liquid food contribution***

As previously explained potable alcohol application does not contribute to the dietary exposure, where brewing is the only application contributing to liquid food exposure for the Beta-glucanase BglS.

| | | |
|---|---|---|
| *Beverage (non milk) intake* | 100.00 | ml/kg bw/day |
| *Processed beverage intake (25%)* | 25.00 | ml/kg bw/day |

The maximum daily intake of Beta-glucanase BglS from brewing and potable alcohol is:

| | | |
|---|---|---|
| **TMDI total** | **155.55** | **µg 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.

Margin of Safety = NOAEL (mg/kg/day) from 90-day / Human cumulative exposure (mg/kg/day)

Margin of Safety = $\frac{\text{1000 mg TOS/kg bw/day}}{\text{0.156 mg TOS/kg bw/day}}$

**Margin of Safety = 6429**

7.4.3 Conclusion

The safety of Beta-glucanase BglS is assessed in a battery of toxicology studies investigating its irritation, acute ingestion, genotoxic and systemic toxicity potential. Beta-glucanase BglS is not an eye and skin irritant (Note: these studies were done as part of assessing worker safety). Beta-glucanase BglS is not acutely toxic by ingestion. A battery of genotoxicity assays was conducted and under the conditions of these assays Beta-glucanase BglS is not a mutagen, a clastogen, or an aneugen.

Daily administration of Beta-glucanase BglS by gavage for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at 1000 mg TOS/kg bw/day (equivalent to 977 mg Total Protein/kg bw/day).

Even under the worst case scenario that Beta-glucanase BglS is applied at the maximum rate and the enzyme is not destroyed and/or removed during processing, the use of Beta-glucanase BglS in brewing and manufacture of potable alcohol is not expected to result in adverse effects to humans. Under the conditions of these safety/toxicology studies, a margin of safety of 6429 exists between the systemic NOAEL of 1000 mg TOS/kg bw/day and the estimated maximum daily human cumulative exposure level of 0.156 mg TOS/kg bw/day.

## 8. BASIS FOR GENERAL RECOGNITION OF SAFETY

As noted in the Safety sections above, *B. subtilis* and enzyme preparations derived there from, including alpha-amylase, cellulase, beta-glucanase and protease enzyme preparations, are well recognized by qualified experts as being safe. Published literature, government laws and regulations, reviews by expert panels such as FAO/WHO JECFA, as well as DuPont Industrial Biosciences' (legacy Danisco) own published and unpublished safety studies and GRAS determinations, support such a conclusion.

*B. subtilis* 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.

Using the decision tree of Pariza and Johnson (2001) the Beta-glucanase BglS enzyme preparation was determined to be acceptable for the proposed uses (Appendix 6). For verification whether the NOAEL is sufficient to support a 100- fold safety margin in the intended uses, the safety studies conducted on the Beta-glucanase BglS produced by the production strain described

in this submission established a NOAEL at 1000 mg TOS/kg bw. Based on a worst-case scenario, the use of Beta-glucanase BglS in brewing and manufacture of potable alcohol application is not expected to result in adverse effects to humans. A safety margin of 6429 exists between the established NOAEL and the estimated worst case maximum daily human cumulative exposure level.

Based on the available data from the literature and generated by DuPont Industrial Biosciences, the company has concluded that Beta-glucanase BglS from *B. subtilis* (CF 624B-1) is safe and suitable for use in brewing and potable alcohol. In addition, the safety determination, including construction of the production organism, the production process and materials, and safety of the product, was reviewed by GRAS expert Dr. Michael Pariza who concurred with the company's conclusion that the product is GRAS (see Appendix 7) for its intended uses.

## 9. LIST OF APPENDICES

Appendix 1. 21CFR170.30

Appendix 2. Amino Acid Sequence of Beta-glucanase BglS

Appendix 3. BglS beta-glucanase production process

Appendix 4. Certificates of Analysis, representative lots

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

Appendix 6. Analysis of Safety Based on Pariza and Johnson Decision Tree

Appendix 7. GRAS Concurrence Letter from Dr. Pariza

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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]

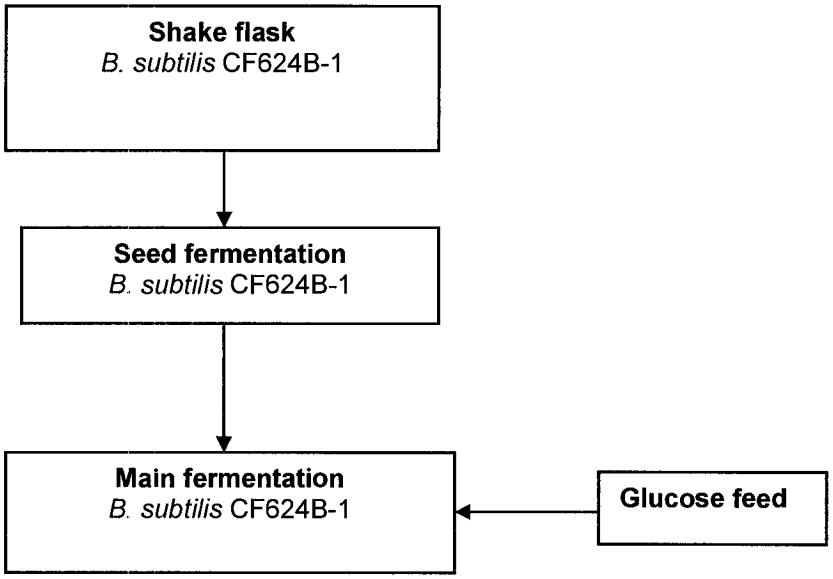
**Appendix 2 Amino Acid sequence of *B. subtilis* Beta-glucanase Bgls**

QTGGSFFDPFNGYNSGFWQKADGYSNGNMFNCTWRANNVSMTSLGEMRLALTSPAYN
KFDCGENRSVQTYGYGLYEVRMKPAKNTGIVSSFFTYTGPTDGTPWDEIDIEFLGKDTT
KVQFNYYTNGAGNHEKIVDLGFDAANAYHTYAFDWQPNSIKWYVDGQLKHTATNQIP
TTPGKIMMNLWNGTGVDEWLGSYNGVNPLYAHYDWVRYTKK

**Appendix 3: Beta-glucanase BglS production processes**

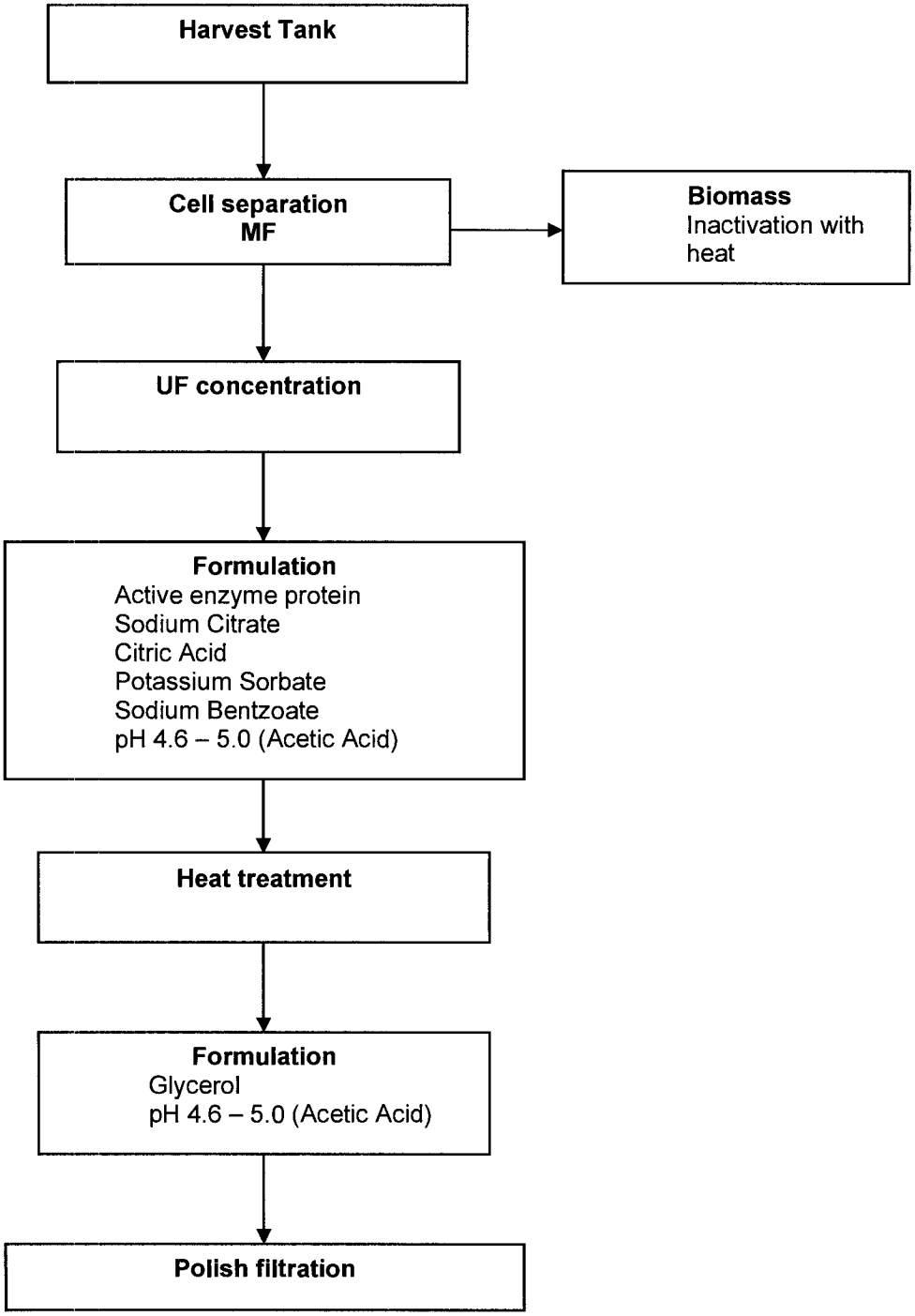
# BglS Production Process Description

## Bgls Fermentation process



GRN
Beta-glucanase from *Bacillus subtilis*
DuPont Industrial Biosciences

## BglS Recovery and formulation process



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**Appendix 4: Certificate of Analysis, Tox Lot**

GRN
Beta-glucanase from *Bacillus subtilis*
DuPont Industrial Biosciences

# CERTIFICATE OF ANALYSIS

925 Page Mill Road · Palo Alto, CA 94304-1013 USA · +1.650.846.7500 tel · www.dupont.com

Name of Test Article: Brew 2 BgIS (beta glucanase)

Production/Strain Name: *Bacillus subtilis* CF 624B-1 strain

Production Site:

Genencor International Culture Collection Number: GICC 03426

Designation of Lot Tested: 20128099

Description: Fermentation liquid – clear brown

Expiration Date: Stable for at least 2 years from date of issuance when stored frozen

***All of the analytical studies listed below were conducted in accordance with GLP regulations and ISO 9002 standards.***

**RESULTS:**

1. Activity: 160365 BBU/g
   (Activity completed by BAMQC per R-SOP-AL-206 (BgIS Cellulase Activity Determination by BGL-DNS)

2. Total and TCA Protein
   The samples were measured for TCA and total protein by nitrogen analysis (with a conversion factor of 6.25 g protein/g nitrogen – Assays conducted by BAMQC per R-SOP-AL-070)

   Total Proteins: 102.29 mg/ml
   TCA Proteins: 69.80 mg/ml
   Active Proteins: 57.0 mg/g
   (Active protein was calculated using a specific activity conversion of 2814 BBU/mg active protein)
   % Total Organic Solids: 10.16 %
   (100% – moisture% – ash%) (100% - 89.03 % - 0.81%)
   (Analysis completed by Silliker Laboratories)

3. Specific gravity: 1.03 g/ml

4. pH: 6.57

5. Microbial analysis: Microbial analysis conducted by Genencor (Rochester, NY) or by outside laboratories.

| Analysis | Results |
|---|---|
| Total viable count | < 4 CFU/ml |
| Coliform | < 1CFU/ml |
| E. Coli | negative |
| Salmonella | negative |
| Staphylococcus aureus | negative |
| Production strain | negative |
| Anaerobic sulfite reducer | negative |
| Lactic acid bacteria | < 1 CFU/ml |

6. Mycotoxin analysis: N/A

7. Heavy metals analysis (analysis completed by BAMQC using Inductively Coupled Plasma-Optical Emission Spectrometry or Direct Mercury Analysis, with the exception of Mercury, which was analyzed by Silliker Laboratories)

| Analysis | Results |
|---|---|
| Heavy metals as Pb | < 30 ppm |
| Arsenic | < 3 ppm |
| Lead | < 5 ppm |
| Mercury | < 0.5 ppm |
| Cadmium | < 0.5 ppm |

8. Stability Data (activity stability analyzed in Rochester under different conditions)

Room Temperature (25° C) (all activity units are reported in /g)

| Sample | T = 0 | T = 5 hours | % of T = 0 |
|---|---|---|---|
| 20128099 (straight) | 166589 | 163256 | 98.0 |
| 20128099 (diluted ½ in water) | 87574 | 86013 | 98.2 |
| 20128099 (diluted ¼ in water) | 44377 | 43515 | 98.1 |

Refrigerated (4°C)

| T = 0 | T = 4 days | % of T = 0 | T = 7 days | % of T = 0 |
|---|---|---|---|---|
| 162094 | 166589 | 102.8 | 153002 | 94.4 |

BEST ORIGINAL COPY

Freezer (-20°C)

| T = 0 | T = 30 days | % of T = 0 | T = 60 days | % of T = 0 | T = 90 days | % of T = 0 |
|---|---|---|---|---|---|---|
| 162094 | 158241 | 97.6 | 164230 | 101.3 | 174918 | 107.9 |

(b) (6)

..... Date: 2/21/13

*Christine Rechichi*
*BioAnalytical Group*
*Industrial Biosciences Division*

(b) (6)

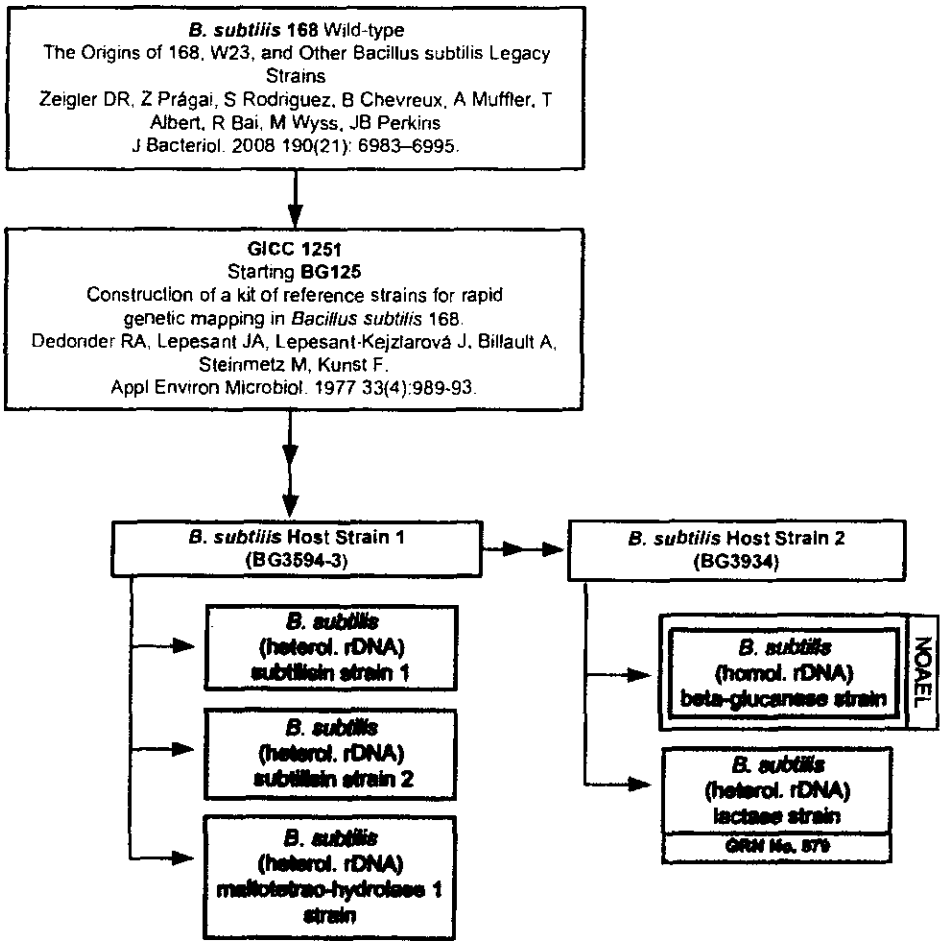
..... Date: 2/22/2013

*Quang Q. Bui*
*Product Stewardship & Regulatory*
*Industrial Biosciences Division*

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

## Safe Strain Lineage: *Bacillus subtilis*

**The donor organism for each individual strain is presented in the respective boxes.**




All commercial enzymes derived from this Safe Strain Lineage were determined to be GRAS for their intended use, with the GRAS Notice reviewed by the US FDA for the enzyme from the strain designated with a green horizontal banner indicating the GRAS Notice number.

The subject strain of this submission is the beta-glucanase producing strain indicated by the yellow border.

The safety of the beta-glucanase enzyme is fully supported by repeated testing of other enzymes produced by members of this Safe Strain Lineage. The orange-colored boxes indicate strains for which we conducted toxicology tests.
The NOAEL for this beta-glucanase is used to calculate its safety margin in the intended uses.

GRN
Beta-glucanase from *Bacillus subtilis*
DuPont Industrial Biosciences

**Toxicology Test Summaries**

The safety of 6 Genencor *Bacillus subtilis* strains and 5 enzyme preparations derived from recombinant production strains were assessed in a number of toxicology tests as shown in the table below. All strains tested were found to be non-cytotoxic/pathogenic and all enzyme preparations were found to be non-toxic, non-mutagenic and not clastogenic.

| PRODUCTION ORGANISM | ENZYME PREPARATION | TOXICOLOGY TEST | RESULT |
|---|---|---|---|
| ***B. subtilis*** **(homol. rDNA) Subtilisin Strain 2** | **Subtilisin** | Cytotoxicity study, Chinese hamster ovary | Non-cytotoxic |
| | | 90-day subchronic study, rats | No adverse effects detected; NOAEL 50,000 ppm |
| ***B. subtilis*** **Host Strain 1 (BG3594-3)** | **Host strain** | Cytotoxicity study, Chinese hamster ovary | Non-cytotoxic |
| ***B. subtilis*** **(heterol. rDNA) Maltotetrao-hydrolase Strain** | **Maltotetrao-hydrolase** | Acute oral toxicity in rats | No signs of toxicity at 2000 mg total protein/kg bw |
| | | 91-day subchronic study, rats | No adverse effects detected, NOAEL = 17.5 mg enzyme protein / kg bw / day |
| | | Ames test | Non-mutagenic |
| | | *In vitro* chromosome assay, human lymphocytes | Non-clastogenic |
| ***B. subtilis*** **(heterol. rDNA) Subtilisin Strain 1** | **Subtilisin** | 90-day subchronic oral study, rats | NOAEL established at highest dose, 420 mg total protein/kg bw/day or 480.6 mg TOS/kg bw/day |
| ***B. subtilis*** **(homol. rDNA) Beta-glucanase Strain** | **Beta-glucanase (Subject of this notice)** | Dermal irritation | Non-irritant |
| | | Eye Irritation | Non-irritant |
| | | Ames assay | Non-mutagenic |
| | | Chromosomal aberration | Non-clastogenic |
| | | 90-day subchronic study | NOAEL established at 1000 mg TOS/kg bw/day or 977.5 mg total protein/kg bw/day |
| ***B. subtilis*** **(heterol. rDNA) Lactase Strain** | **Lactase** | Dermal irritation | Non-irritant |
| | | Eye Irritation | Non-irritant |
| | | Acute oral toxicity in rats | No signs of toxicity at 5000 mg total protein/kg bw |
| | | Ames test | Non-mutagenic |
| | | *In vitro* chromosome assay, human lymphocytes | Non-clastogenic |
| | | 90-day subchronic oral study, rats | No adverse effects detected, NOAEL established at highest dose, 1000mg total protein/kg bw/day or 1416.4mg TOS (total organic solid)/kg bw/day |

**Study Summaries**

***B. subtilis* (homol. rDNA) Subtilisin Strain 2 - (Subtilisin)**

In a 90-day oral feeding study, groups of rats were fed with a concentrated Subtilisin enzyme solution produced from Genencor *B. subtilis* Subtilisin Strain 2 at 0, 5000, 15000 or 50000 ppm in the diet for 90 consecutive days. Feed consumption and body weight gain were significantly decreased during the first few weeks of treatment and were related to poor palatability. By study termination, all treated groups had comparable weight gain and feed consumption as the control group. There were no overt signs of toxicity throughout the entire study. One control animal died and the cause of death was not known. At necropsy, dose-related increases in salivary gland weights were noted in both male and female treated groups. These findings were related to hypertrophy of the serous acinar cells of the sub-mandibular salivary glands in both male and female treated rats. The presence of hypertrophy of the sub-mandibular salivary glands was determined not as an adverse effect but rather as a physiological adaptation and local response to continuous exposure to a Subtilisin in the diet. The effect was expected due to the irritant property of Subtilisin. There were no other abnormal histopathologic findings found in the treated groups. No treatment related effects were noted in hematology, clinical chemistry, ophthalmology, and urinalysis. Consequently, the systemic NOAEL was established at 50,000 ppm (5% in the diet).

In a cytotoxicity study using Chinese hamster ovary, two Genencor *B. subtilis* strains direct descendants of strain 168 (BG 125 and Subtilisin Strain 2 which is a GM descendant of BG125) were tested for cytotoxicity and effects on mitochondrial activity (through toxin production). $IC_{50}$ (inhibition constant 50) values were calculated and compared to both positive and vehicle controls. Under the conditions of this assay, both Genencor *B. subtilis* strains (Subtilisin Strain 2 and BG125) were not cytotoxic and did not interfere with mitochondrial activity suggesting that toxin was not produced by either Genencor *B. subtilis*.

**REFERENCES:**

Pharmakon, report No. PH-470-GNC 001-94. 90-day feeding study, rats. 1994.

Huntingdon Life Sciences report No. GNC 001B/021251. Cytotoxicity study. 2002

***B. subtilis* Host Strain 1- BG125 (BG3594-3)**

**Cytotoxicity study in Chinese hamster ovary.**

In a cytotoxicity study using Chinese hamster ovary, two Genencor *B. subtilis* strains direct descendants of strain 168 (BG125 and Subtilisin Strain 2 which is a GM descendant of BG125) were tested for cytotoxicity and effects on mitochondrial activity (through toxin production). $IC_{50}$ (inhibition constant 50) values were calculated and compared to both positive and vehicle controls. Under the conditions of this assay, both Genencor *B. subtilis* strains (Subtilisin Strain 2 and BG125) were not cytotoxic and did not interfere with mitochondrial activity suggesting that toxin was not produced by either Genencor *B. subtilis*.

**REFERENCES:**

Huntingdon Life Sciences report No. GNC 001B/021251. Cytotoxicity, 2002

***B. subtilis* (heterol. rDNA) Maltotetrao-hydrolase Strain – ( Maltotetrao-hydrolase)**

**Acute Oral Toxicity in the Rat**

The objective of this study is to assess the acute toxicity of Maltotetrao-hydrolase for hazard assessment and ranking purpose. A single dose of 2000 mg total protein/kg bw (equivalent to 110.8 mg enzyme protein/kg bw) was given to either a single female rat (sighting study) or a group of 4 female rats (main study). All animals were observed daily for 14 consecutive days. Body weights were recorded at various intervals throughout the 14-day period. All animals were killed at study end and a necropsy performed.

No deaths were found in both the sighting and main studies. Piloerection was noted but no overt signs of toxicity were found throughout the entire investigation. There were no adverse effects on body weights, food consumption and pathological examinations.

This test was not intended to establish an exact $LD_{50}$, hence a limit test of 2000 mg/kg was used. Under the conditions of this assay, Maltotetrao-hydrolase is practically non-toxic by ingestion to rats as evidenced by the lack of mortality noted at 2000 mg total protein/kg bw (equivalent to greater than 110.8 mg enzyme protein/kg bw). Based on the $LD_{50}$ value, Maltotetrao-hydrolase would be classified in the GHS Category 5.

**Bacterial Reverse Mutation Assay – Ames assay**

The objective of this assay is to assess the potential of Maltotetrao-hydrolase to induce point mutation (frame-shift and base-pair) in five bacterial tester strains: *Salmonella typhimurium* TA 98, TA 100, TA 102, TA 1535 and TA 1537. The test material was tested both in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver S-9). The doses selected for the main assay were based on results from a preliminary toxicity test. The positive controls used for assays without S-9 mix were sodium azide, 2-nitrofluorene and cumene hydroperoxide and the positive control used for assays with S-9 mix was 2-aminoanthracene. Triplicate plates were run for each dose level and the entire assay was repeated twice. This assay was conducted in accordance with OECD guideline No. 471 (Bacterial Reverse Mutation Test).

Based on the results of the preliminary assay, the following dose levels were selected for the main assay: 52.4, 168, 524, 1680 and 5240 ug total proteins/plate. The highest dose used, 5240 ug total proteins/plate, exceeded the maximum dose level of 5000 ug/plate required by OECD guideline No. 471.

The mutagenic potential of Maltotetrao-hydrolase was first assayed with the pre-incubation method. In this assay, five dose levels of Maltotetrao-hydrolase were mixed with a bacterial suspension. Top agar was added, mixed and the mixtures were spread on selective agar plates. The plates were then incubated

at 37°C for 72 hours and the number of revertant colonies on each plate was counted. In light of the presence and growth of the background lawn of non-revertant bacteria, the pre-incubation method was considered inappropriate to assess the mutagenic potential of Maltotetrao-hydrolase. The growth of the background lawn was related to the presence of the amino acid histidine in the test material. The treat and plate method was selected instead.

In the treat and plate method, various concentrations of Maltotetrao-hydrolase were mixed with a concentrated bacterial suspension and nutrient broth. After a period of incubation, bacteria were separated by sedimentation, re-suspended with buffer and mixed with top agar. The whole process was repeated twice and then the mixture was spread on selective agar plates. The plates were incubated at 37°C for 72 hours and the number of revertant colonies on each plate was counted. Triplicate plates were used for each dose level and the whole assay was repeated twice.

Cytotoxicity was demonstrated at the highest dose tested (5240 ug/plate) in strain TA 1537 both in the presence and absence of metabolic activation. However, cytotoxic effects were not found in all other strains and at any other dose levels. There was a statistical increase in the number of revertant colonies at the 5240 ug/plate dose level with the TA 102 strain in the absence of S-9 mix. However, no statistical differences were noted in the second trial with TA 102 both with and without 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.

All the bacterial strains used in this assay carry mutations in the histidine operon and the reliability and sensitivity of the pre-incubation assay would be compromised if the amino acid histidine is present. Dose-related increases in the growth of the background lawn of non-revertant colonies in the pre-incubation assay were due to the presence of histidine in Maltotetrao-hydrolase. Therefore, the pre-incubation assay was considered not suitable for this enzyme. The compromising effect of histidine-containing enzyme is not uncommon and false positive results from the Ames tests have been reported (Pariza and Johnson, 2001).

In the treat and plate method, cytotoxicity was demonstrated with TA 1537 at the highest dose tested (5240 ug/plate). This finding was expected since it exceeded the maximum dose required by OECD guideline (5000 ug/plate) and demonstrated the suitability of the treat and plate method for Maltotetrao-hydrolase. No cytotoxic effects were observed at all other dose levels and tester strains. The increase in the number of revertant colonies with TA 102 at 5240 ug/plate was not considered as biologically significant since the findings were not confirmed in the repeat assay and were not evident in TA 100, the second strain used for detection of base-pair substitution.

Under the conditions of this assay, there is no evidence to suggest that Maltotetrao-hydrolase is a mutagen in the Ames assay.

***In vitro* Chromosomal Aberration Test**

The objective of this assay is to investigate the potential of Maltotetrao-hydrolase to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes).

Maltotetrao-hydrolase was mixed with cultures of human peripheral lymphocytes both in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver S-9). This test was performed in two assays. In the first assay, all cultures (with and without S-9 mix) were treated for 3 hours and harvested 20 hours after the start of treatment. In the second assay, S-9 mix cultures were treated for 3 hours whereas non-activated cultures were treated for 20 hours continuously. All cultures were harvested 20 hours after the start of treatment. Two hours prior to the scheduled cell harvest, Demecolcine was added to all cultures. Metaphase cells were harvested by centrifugation and re-suspended in appropriate medium. The cells were then fixed on slides and stained. For each culture, 100 metaphases were examined for the presence or absence of chromosomal aberrations. Daunomycin was used as the positive control in the non-activated assay and cyclophosphamide was the positive control in the activated assay.

This assay was conducted in accordance with OECD guideline No. 473 (*In vitro* Mammalian chromosome aberration test).

In the preliminary dose-range assay, cytotoxicity was observed at 5240 ug/ml in activated cultures and at 5240, 2620 and 1310 ug/ml in non-activated cultures. Based on these results, the following dose levels were selected for the main assays: 164, 328, 655, 1310, 2620 and 5240 ug/ml for non-activated cultures and 655, 1310, 2620 and 5240 ug/ml for activated cultures.

A reduction in mitotic index was noted at the 5240 ug/ml dose level in both activated and non-activated cultures. No biologically or statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with Maltotetrao-hydrolase both in the presence and absence of metabolic activation (S-9 mix). Significant increases in aberrant metaphases were demonstrated with the positive controls.

A reduction in mitotic index was noted at the 5240 ug/ml. However, this finding was not unexpected since this dose exceeded the maximum dose recommended by OECD guideline, i.e. 5000 ug/ml. There were no adverse effects noted at all other dose levels.

Under the conditions of this test, there is no evidence to suggest that Maltotetrao-hydrolase iinduces chromosomal aberrations (both structural and numerical) in mammalian cells both in the presence and absence of metabolic activation.

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

The objective of this study was to investigate the potential of Maltotetrao-hydrolase to induce systemic toxicity after repeated daily oral administration.

Groups of 10 Sprague Dawley rats/sex each were gavaged daily with 0, 78.5, 157.05 or 314.1 mg total protein/kg body weight in a constant aqueous volume of 10 ml/kg body weight. These doses corresponded to, respectively, 0, 4.3, 8.7 or 17.5 mg enzyme protein/kg bw/day and 0, 127.4, 254.7 or 509.4 mg TOS/kg bw/day.

All animals were observed daily for mortality and signs of morbidity. Animals of the same sex were pair-housed in transparent polycarbonate cages with softwood sawdust as bedding and had access to water (via bottle) and feed *ad libitum*. All groups were housed under controlled temperature, humidity and lightning conditions.

Body weight and feed consumption were recorded weekly. Ophthalmologic examination was performed on all animals prior to study initiation and in the control and high dose groups at study termination. During week 11, a functional observation battery and behavior observation were performed on all animals. Hematology and clinical chemistry were conducted at study termination prior to a necropsy, which was performed on all groups. After a thorough macroscopic examination, selected organs were removed, weighed and processed for future histopathologic examination. Microscopic examination of selected organs was conducted first on control and high dose animals. If a questionable finding was noted, the microscopic examination would be extended to the low and mid dose groups.

This study was conducted in accordance with OECD guideline No. 408.

There were no treatment-related deaths in this study. Two animals (one control female and one mid-dose female) were killed *in extremis* due to intubation error confirmed by necropsy findings of perforated esophagus and reddish lungs.

There were no biological or statistical differences between the control and treated groups with respect to feed consumption, body weights, body weight gains, hematology, clinical chemistry, absolute and relative organ weights, clinical observations, and ophthalmologic examinations. There were no treatment-related histopathologic changes. In the open-field testing, there were statistically significant decreases in ambulatory movements in treated females when compared with the concurrent control. However, similar effects were not found in treated males.

Two animals were killed *in extremis* in this study, one control female and one mid dose female, due to procedural error. The loss of two animals did not affect the integrity and validity of the study. Throughout the entire study, there were no treatment related effects in all parameters investigated, from clinical observations to histopathologic examinations. The statistical differences in ambulatory movements noted in treated females were not considered as biologically significant since the differences were within the standard deviations of the historical control data collected at the testing facility. Further, the differences did not follow a clear dose response relationship. Under the conditions of this assay, it can be concluded that oral gavage of Maltotetrao-hydrolase for 90 continuous days did not result in systemic toxicity in the rat. The NOAEL (No observed adverse effect level) is established at greater than 314.1 mg total protein/kg bw/day equivalent to 17.5 mg enzyme protein/kg bw/day or 509.4 mg TOS/kg bw/day.

**REFERENCES:**

Scantox Study No. 53314, Acute oral toxicity study in the rat, December 15, 2003.

Scantox Study No. 53315, Ames Test, March 10, 2004.

Scantox Study No. 53316, *In vitro* mammalian chromosome aberration test performed with human lymphocytes, March 10, 2004

Scantox Study No. 53156, A 13-week oral (gavage) toxicity study in rats. March 11, 2004.

***B. subtilis* (heterol. rDNA) Subtilisin Strain 1 (Subtilisin)**

**13wk Oral (Gavage) Toxicity Study in Rats**

To assess the safety of the Subtilisin enzyme preparation derived from this strain, a 90-day oral (gavage) study was investigated at CiToxLAB Scantox (Denmark) and the results are evaluated, interpreted and assessed below. The test material (UFC) used in all toxicology investigations has the following characteristic (reference Appendix 8):

| | |
|---|---|
| Lot No.: | 6202401 |
| Physical: | Fermentation liquid, brown |
| pH: | 6.77 |
| Specific gravity: | 1.08 g/ml |
| Total protein: | 193.40 mg/ml |
| TOS: | 22.13% |

The objective of this study was to investigate the potential of the Subtilisin to induce systemic toxicity after repeated daily oral administration to SPF Sprague Dawley rats (Taconic M&B, Denmark) of both sexes for 90 consecutive days. Groups of 10 rats/sex each were gavaged daily with 0 (0.9% saline), 105, 210 or 420 mg total protein/kg body weight in a constant volume of 5 ml/kg body weight corresponding to 120.2, 240.3 or 480.6 mg TOS/kg bw/day, respectively.

Animals of the same sex were pair-housed in transparent polycarbonate cages with softwood sawdust as bedding and had access to water (via bottle) and feed *ad libitum*. For environmental enrichment, the animals were provided a supply of Aspen Wood Wool at each change of bedding. All groups were housed under controlled temperature, humidity and lightning 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. Hematology was 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 13 for the control and high dose groups. Clinical chemistry was evaluated at study termination prior to necropsy on all groups. After a thorough macroscopic examination, selected organs were removed, weighed and processed for future histopathologic examination. Microscopic examination was conducted on selected organs from control and high dose animals.

This study was conducted in accordance with OECD guideline No. 408 (September 1998) and EPA Guideline OPPTS 870.3100 (August 1998) and complied with OECD Principles of GLP (as revised in 1997) and all subsequent OECD consensus documents.

Four animals were found dead - two males and one female in the low dose group and one high dose male. Blood, blood clots or reddish watery fluid was observed in the chest cavity at necropsy indicating mis-dosing of fluid into the chest cavity. One mid-dose female was killed in a moribund condition and at the microscopic examination inflammation of the lungs and larynx was observed, correlating well with the suspicion of a dosing accident. These mortalities were therefore considered as procedural errors (gavage errors) and not as treatment related.

A slight decrease in body weight gain was observed for the high dose males. However, as this finding was within the normal historical range, it was not considered of toxicological importance.

Administration of the Subtilisin for 90 consecutive days did not result in any treatment related effects on clinical examination, feed consumption, water consumption, ophthalmoscopic examination, urinalysis, clinical chemistry, hematology and coagulation parameters. No treatment related effects were noted in the functional observation battery and stimuli-induced tests. At necropsy, at the organ weight analysis and at the histopathologic examination, no treatment related findings were recorded.

In conclusion, daily administration by oral gavage of the Subtilisin (from *Bacillus subtilis)* to Sprague Dawley rats for 13 weeks at dosages of 0, 105, 210 and 420 mg total protein/kg/day did not cause any test item related changes.

Consequently, in this study, the NOAEL (no observed adverse effect level) was 420 mg total protein/kg/day (corresponding to 480.6 mg TOS/kg bw/day).

In this study, five animals died. However, all five mortalities were not considered as treatment-related but rather due to gavage error. Therefore, daily administration of the Subtilisin by oral gavage for 90 consecutive days did not result in adverse 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, 420 mg total protein/kg bw/day corresponding to 480.6 mg TOS/kg bw/day.

**REFERENCES:**

Scantox Study No. 73796, A 13-week oral (gavage) toxicity study in rats, November 23, 2011.

***B. subtilis* (homol. rDNA) Beta-glucanase Strain (Beta-glucanase)**

In dermal and eye irritation studies, no deaths or overt signs of toxicity were observed. No effects on feed consumption and weight gain were recorded. No dermal or eye irritation was observed at any dose site. According to the United Nations Globally Harmonized System of Classification and Labeling of Chemicals (GHS), under the conditions of these studies, classification is not required.

The genotoxicity potential of beta-glucanase was investigated in a bacterial reverse mutation assay (Ames assay) and an *in vitro* chromosomal aberration assay using human peripheral blood lymphocytes. Under the conditions of the mutagenicity assays, beta-glucanase is not a mutagen or clastogen in the presence and absence of metabolic activation.

The systemic toxicity potential of beta-glucanase after repeated administration was investigated in a sub-chronic oral gavage study. Beta-glucanase was administered 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 (0.9% saline), 100, 300 or 1000 mg TOS/kg bw/day. The dosing volume was 10 ml/kg bw/day. 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. This study was conducted in accordance with OECD guideline No. 408 (September 1998).

Daily administration of beta-glucanase by gavage for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at 1000 mg TOS/kg bw/day (equivalent to 977.5 mg total protein/kg bw/day).

Eurofins PSL: H-30535 Primary skin irritation in rabbits; Report No. 35783 – Dupont No. 20178-1008; Final report dated March 20, 2013.

**REFERENCES:**

Eurofins PSL: H-30535 Primary eye irritation in rabbits; Report No. 35782 – Dupont No. 20178-602; Final report dated March 20, 2013.

BioReliance: H-30535: Bacterial reverse mutation assay; Report No. AD64LW.507001.BTL; Dupont No. 20178-513; Final report dated April 3, 2013.

BioReliance: H-30535: In vitro mammalian chromosome aberration test in human peripheral blood lymphocytes; Report No. AD64LW.341.BTL; Dupont No. 20178-544; Final report dated May 10, 2013.

MPI Research: H-30535: Subchronic toxicity 90 day oral gavage study in rats; Report No. 125-172; Dupont No. 20178-1026; Final report dated November 27, 2013.

National Institute on Alcohol Abuse and Alcoholism, US Department of Human Health Service, National Institutes of Health: Surveillance Report # 92, Apparent Per Capita Alcohol Consumption: National, State and Regional Trends 1977-2009, August 2011.

***B. subtilis* (heterol. rDNA) Lactase Strain – (Lactase)**

To assess the safety of the lactase, different endpoints of toxicity were investigated and the results are evaluated, interpreted and assessed in this document. The test material, an ultra-filtrate concentrate (UFC) used in all toxicology investigations, has the following characteristics:

| | |
|---|---|
| Lot No.: | 20138117 |
| Physical: | Fermentation liquid, brown |
| Enzyme activity: | 1369 LAU/g |
| pH: | 7.21 |
| Specific gravity: | 1.058 g/ml |
| Total protein: | 133.13 mg/ml |
| TOS: | 17.83 % |

**Acute dermal irritation study in rabbits (sequential approach)**

The objective of this study was to assess the local irritant effect of the lactase. This study was conducted according to the method recommended in the OECD Guideline No. 404, April 2002. This study was conducted in a stepwise manner. Initially, 5/10 of a milliliter of the test substance was applied to the skin of one rabbit for 4 hours. Since no dermal irritation was noted in this rabbit, a confirmatory test was completed with two additional healthy rabbits. Approximately 24 hours prior to application, all animals were prepared by clipping the dorsal area and the trunk. Five tenth of one milliliter was applied to one 6 $cm^2$ intact dose site and covered with 1 inch x 1 inch, 4-ply gauze pad. The pad and entire trunk of each animal were then wrapped with semi-occlusive tape. Elizabethan collars were placed on each rabbit. After 4 hours of exposure, the pads and collars were removed and the test sites were cleansed of any residual test substance. The grade of skin reaction was scored according to the Draize's scoring system immediately following patch removal and at 30-60 minutes, 24, 48 and 72 hours after patch removal.

No deaths or overt signs of toxicity were observed in this study. No effects on feed consumption and weight gain were recorded. No dermal irritation was observed at any dose site during the study. According to the provisions of Directive 67/548/EEC amended by Commission Directive 2001/59/EC of 6 August 2001, Annex VI, classification is not required. According to the United Nations Globally Harmonized System of Classification and Labeling of Chemicals (GHS), under the conditions of this study, classification is not required.

**Acute Eye Irritation/Corrosion Study in the Rabbit**

The objective of this study was to assess the ocular irritation potential of the lactase. This study was conducted according to the method recommended in the OECD Guideline No. 405, 24 April 2002 and evaluated according to the Commission Directive 2001/59/EC of 6 August 2001.

The study was conducted in a stepwise fashion. Initially, 1/10 of a milliliter of the test substance was instilled into the conjunctival sac of the right eye of one rabbit. The left eye remained untreated and served as control. The grade of ocular reaction was recorded at 1, 24, 48 and 72 hours post instillation and evaluated by the method of Draize *et al.* At the 24-hour reading, fluorescein was instilled and then rinsed with 0.9% NaCl. The eye was then examined with an UV-light to detect corneal damage. Since there was no significant ocular irritation noted in this rabbit, the test was repeated on two additional rabbits by the same procedure.

No ocular irritation was noted in all three rabbits. According to the provisions of Directive 67/548/EEC amended by Commission Directive 2001/59/EC of 6 August 2001, Annex VI, classification is not required. According to the United Nations Globally Harmonized System of Classification and Labeling of Chemicals (GHS), under the conditions of this study, classification is not required.

**Acute Oral Toxicity in Rats – Up and Down Procedure**

The objective of this study is to determine the oral toxicity of the lactase given by a single administration using the Up and Down procedure. The study was conducted according to OECD Guideline No. 425 (2008). A single dose of 5000 mg/kg was administered by oral gavage to fasted female rats. Since the first animal survived, two additional animals were dosed simultaneously after a minimum of 48 hours. All rats were observed for mortality, body weight, and clinical signs for 14 days after dosing. All rats were necropsied at the end of the 14 day.

No incidents of mortality, body weight loss, or clinical signs were observed. No gross lesions were noted at necropsy. Under the conditions of this assay, the oral $LD_{50}$ for the lactase in female rats was greater than 5000 mg/kg bw/day. According to the provisions of Directive 67/548/EEC amended by Commission Directive 2001/59/EC of 6 August 2001, Annex VI, classification is not required. According to the United Nations Globally Harmonized System of Classification and Labeling of Chemicals (GHS), under the conditions of this study, classification is not required.

**Bacterial Reverse Mutation Assay – Ames assay.**

The objective of this assay was to assess the potential of the lactase 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 *Escherischia coli* strain WP2 *uvr*A. 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. This assay was conducted in accordance with OECD guideline No. 471 (1997).

In the screening assay, lactase was not toxic to the 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 of S-9 mix or with tester strains TA1535, TA1537 and WP2 *uvr*A in the absence of S-9 mix. Toxicity was observed beginning at 1500 µg TP/plate with strains TA98 and TA 1537 in the absence of S-9 mix. 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. Neither precipitate nor toxicity was observed. 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. Under the conditions of this assay, lactase has not shown any evidence of mutagenic activity in the Ames assay in both presence and absence of metabolic activation.

***In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes.**

The objective of this assay was to investigate the potential of the lactase 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.

The lactase 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. Ten concentrations of the lactase were used in the preliminary assay and at least 4 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 preliminary assay, all cultures with or without S-9 mix were treated for 4 hours and continuously for 20 hours in the absence of S-9 mix. In the definitive assay, cultures with and without S-9 mix were exposed to the test article for 4 hours, and continuously for 20 hours in the absence of S-9 mix. For the preliminary and the definitive assays, cells were collected 20 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.

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. Metaphase analysis (i.e., evaluation of chromosomal aberration) was conducted on at least 200 metaphases for each dose level (100 per duplicate treatment). Cells were scored for both chromatid-type and chromosome-type aberrations. Mitomycin C and cyclophosphamide were used as positive controls for cultures without S9 and cultures with S9, respectively. This assay was conducted in accordance with OECD guideline No. 473 (1977).

In the preliminary assay, the dose levels ranged from 0.5 to 5000 µg TP/ml. Exposure period was 4 hours for both cultures with and without S9 mix, and continuously for 20 hours in the absence of S-9 mix. All cells were harvested after 20 hours after treatment initiation. Visible precipitation of the test material in the culture medium was observed at 5000 µg TP/mL. At the conclusion of the treatment period, hemolysis was observed at dose levels $\geq$ 1500 µg TP/mL in the S-9 mix activated 4-hour and the non-activated 20-hour treatment groups. Substantial toxicity (at least 50% reduction in mitotic index relative to the vehicle control) was observed at any dose levels $\geq$ 1500 µg/mL in the non-activated 4-hour exposure group and at dose levels $\geq$ 150 µg TP/mL in the non-activated 20-hour exposure group. Based on those findings, dose levels ranging from 100 to 1500 µg/ml were used in the definitive assays for the non-activated 4-hour treatment group, from 2 to 550 µg TP/mL for the activated 4-hour treatment group, and from 10 to 200 µg TP/mL for the non-activated 20-hour treatment group.

In the definitive assay, at the conclusion of the treatment period, precipitate was observed in the S-9 mix activated 4-hour exposure group at dose levels $\geq$ 450 µg TP/mL and at dose levels $\geq$ 100 µg TP/mL in the non-activated 20-hour treatment group. Based on these findings, the doses chosen for microscopic analysis ranged from 100 to 500 µg TP/mL for the S-9 mix activated 4-hour exposure group, from 200 to 850 µg TP/mL for the non-activated 4-hour exposure group and from 25 to 100 µg TP/mL for the non-activated 20-hour exposure group.

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.

Under the conditions of this test, the lactase 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. 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. The lactase is not considered as clastogenic.

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

The objective of this study was to investigate the potential of the lactase to induce systemic toxicity after repeated daily oral administration to Crl:CD(SD) rats of both sexes for 90 continuous days. Groups of 10 animals per sex were treated by oral gavage with 0 (deionized water), 100, 300 or 1000 mg total protein (TP)/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. This study was conducted in accordance with OECD guideline No. 408 (September 1998).

No treatment-related deaths were noted during the 13-week period. One control and one mid dose male died following sublingual bleeding for clinical pathology parameters. One mid dose male was found dead on day 55 but was not considered as treatment related. 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.

Daily administration of the lactase by oral gavage to rats at doses of 0, 100, 300 or 1000 mg TP/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, 1000 mg TP/kg bw/day equivalent to 1416.4 mg TOS (total organic solid)/kg bw/day in male and female rats.

The safety of the lactase is assessed in a battery of toxicology studies investigating its genotoxicity and systemic toxicity potential. Under the conditions of the mutagenicity assays, the lactase is not a mutagen or clastogen. Daily administration of the lactase by gavage for 90 continuous days did not result in overt

signs of systemic toxicity. A NOAEL is established at 1000 mg TP/kg bw/day corresponding to 1416.4 mg TOS/kg bw/day.

**REFERENCES:**

PSL: H-30869 Primary skin irritation in rabbits; Report No. 37515 – DuPont No. 20510-603; Final report dated December 04, 2013.

PSL: H-30869 Primary eye irritation in rabbits; Report No. 37514 – DuPont No. 20510-602; Final report dated November 27, 2013.

DuPont Haskell Global Center: H-30869 – Acute oral toxicity in rats – Up and Down Procedure Report No. 20510-834; Final report dated February 24, 2014.

BioReliance: H-30869: Bacterial reverse mutation assay; Report No. AD81WY.507001.BTL; DuPont No. 20510-513; Final report dated February 11, 2014.

BioReliance: H-30869: In vitro mammalian chromosome aberration test in human peripheral blood lymphocytes; Report No. AD81WY.341.BTL; DuPont No. 20510-544; Final report dated February 10, 2014.

DuPont Haskell Global Center: H-30869: Subchronic toxicity 90 day oral gavage study in rats; Report No. 20510-1026, Final report dated August 8, 2014.

## Appendix 6: Pariza Decision Tree on *B subtilis* BglS beta-glucanase

**1. Is the production strain [2] genetically modified? [3,4]**

Yes → go to 2

**2. Is the production strain modified using rDNA techniques?**

Yes → go to 3a

**3a. Do the expressed enzyme product(s) which are encoded by the introduced DNA[5,6] have a history of safe use in food? [7]**

Yes, beta-glucanase has been used for years in food processing and animal feed. The *Bacillus subtilis* beta-glucanase BglS is new as an isolate expressed in *B. subtilis* for use in food processing, but has been present at lower amounts in other enzyme preparations from its donor organism. Additionally, it's GRAS per 21CFR184.1148. → go to 3c.

**3c. Is the test article free of transferable antibiotic resistance gene DNA? [8]**

Chloramphenicol is regarded endogenous to Bacillus, and, once integrated in the genome, not readily transferable to other species. Yes → go to 3e.

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[2] 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

[3] 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.

[4] 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.

[5] IntroducedDNArefers to allDNAsequences 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.

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

[7] 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.

[8] 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 )

**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 → go to 4

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

No, the expression cassette, consisting of the *aprE* promoter beta-glucanase BglS gene and *Bacillus amyloliquefaciens apr* termination and the chloramphenicol resistance marker gene from plasmid pC194 (originally isolated from *S. aureus* but widely recognized to be naturally present in *Bacillus*), is introduced into the host genome, at the site of the endogenous alkaline protease *aprE* gene, without any vector sequences remaining in the final production strain. Go to 6.

**6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?**[9]

Yes, the *Bacillus subtilis* safe lineage is established as presented 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).

**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 *Bacillus subtilis* Beta-glucanase BglS expressed in *Bacillus subtilis* strain CF 624B-1is safe and suitable for use in brewing application, potable alcohol manufacture, and is Generally Recognized as Safe (GRAS) for those uses.

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[9] 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.

GRN
Beta-glucanase from *Bacillus subtilis*
DuPont Industrial Biosciences

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RE: GRAS Opinion on the Intended Uses of DuPont's BglS β-glucanase Enzyme Preparation Derived from *Bacillus subtilis*

Dear Dr. Sewalt,

I have reviewed the information you provided on DuPont's *B. subtilis* β-glucanase enzyme preparation, designated BglS, which is expressed in *B. subtilis* CF 624B-1 (GICC03426), a production strain that has been genetically modified to over-express its own native β-glucanase enzyme gene. The intended use of BglS is as a processing aid to facilitate hydrolysis of beta-glucans and related carbohydrates in brewing and potable alcohol manufacture, where the enzyme is either not present in the final food, or present at trace levels as inactive protein having no function or technical effect.

In evaluating the safety of BglS, I considered the biology of *Bacillus subtilis*; the fact that *B. subtilis* BG125 is both the host strain and source of the wild-type BglS gene; information that you provided regarding the cloning methodology that was utilized; information pertaining to the safe strain lineage within which *B. subtilis* BG125 and *B. subtilis* CF 624B-1 (GICC03426) were developed; the fact that the BglS β-glucanase is present as a side activity in other enzyme preparations that are expressed by *B. subtilis* production strains within the DuPont safe lineage; and other relevant information available in the peer-reviewed scientific literature.

By way of background, *B. subtilis* is a ubiquitous gram positive spore-forming bacterium that is rarely associated with opportunistic infections or food poisoning outbreaks. Many non-pathogenic, non-toxigenic strains of this species are utilized by enzyme manufacturers worldwide to produce enzymes and other products for industrial applications, including human

food and animal feed uses. Carbohydrase and protease enzyme preparations derived from *B. subtilis* have been affirmed as GRAS by the U.S. FDA per 21 CFR 184.1148 and 184.1150, respectively.

DuPont's safe lineage of non-pathogenic, non-toxigenic *B. subtilis* production strains, which includes *B. subtilis* BG125 and *B. subtilis* CF 624B-1 (GICC03426), was developed from the wild-type *B. subtilis* 168 via a series of modifications that included classical mutagenesis, as well as rDNA and protein engineering utilizing techniques and reagents that are appropriate for the development of a safe lineage of food ingredient production microorganisms. The safety of the enzymes from these production strains have been evaluated with various *in vitro* genetic toxicity tests, as well as oral toxicity tests in rats (90-day, 28-day, or acute oral toxicity). Strains within this safe lineage are used to manufacture many food and feed enzymes, including proteases, arylesterase, maltotetraohydrolase, xylanase, cellulase, and β-glucanase. Published literature, government laws and regulations, and DuPont's unpublished safety studies, all support the conclusion that the lineage to which these production strains belong is safe and suitable for use in the development and manufacture of food-grade and feed-grade enzymes. Positive GRAS determination expert opinion letters were received for the following enzymes produced by other strains within this safe lineage: Multifect P300 protease for food and feed (Dr. Pariza to G. Mercer 18 May 1994, and to A. Caddow 1 October 1994), maltotetrahydrolases (SAS 1, 2, & 3 amylase; 27 May 2004, 17 October 2005, & 29 August 2006, respectively from Drs. Pariza, Borzelleca, and Blumenthal) from three strains for baking, and a xylanase for baking (Dr. Pariza to A. Caddow, 28 September 2006).

It should be noted that the BglS β-glucanase is present as a side activity in some of these other enzyme preparations that are expressed by *B. subtilis* production strains in the DuPont safe lineage, for example 1,4-xylanase (hemicellulase) and α-amylase, both of which have undergone exhaustive safety evaluation including testing for acute, genotoxic and subchronic toxicity.

To construct *B. subtilis* CF 624B-1 (GICC03426), multiple copies of the *B. subtilis* BG125 native BglS β-glucanase were introduced back into *B. subtilis* BG125, using cloning techniques and methodologies that are appropriate for use in the genetic modification of production strains for food ingredient manufacture.

Based on a 13-week oral (gavage) study in CD rats, the NOAEL for the BglS beta-glucanase enzyme preparation was determined to be 1000 mg TOS/kg bw/day, equivalent to 977.5 mg total protein/kg bw/day. Consumer exposure to BglS use in brewing and potable alcohol manufacture is estimated to be 0.156 mg TOS/kg bw/day, giving a margin of safety of 6429, well below the 100-fold safety factor that is typically applied to food ingredients. Given the extensive database of safety evaluations and established history of safe use in enzyme manufacture of DuPont's safe lineage of non-pathogenic, non-toxigenic *B. subtilis* production strains, I conclude that the required elements for evaluating the safety of the BglS β-glucanase expressed by *B. subtilis* CF 624B-1 (GICC03426) are met (MW Pariza and EA Johnson, Regulatory Toxicol. Pharmacol. 33: 173-186,2001).

The manufacturing process including the ingredients used for fermentation, extraction and concentration of BglS, and the specifications for BglS, are appropriate for a food ingredient.

Based on the foregoing, I concur with the evaluation made by DuPont that the *B. subtilis* CF 624B-1 (GICC03426) production strain is safe and appropriate to use for the manufacture of food-grade BglS β-glucanase. I further conclude that the BglS β-glucanase enzyme, manufactured in a manner that is consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food-grade specifications, is GRAS (Generally Recognized As Safe) for use as a processing aid to facilitate hydrolysis of beta-glucans and related carbohydrates in brewing and potable alcohol manufacture, where the enzyme is either not present in the final food, or present at trace levels as inactive protein having no function or technical effect.

It is my professional opinion that other qualified experts would also concur in this conclusion.

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,

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Michael W. Pariza
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SUBMISSION END