#1 584

Danisco US Inc.
925 Page Mill Road
Palo Alto, CA 94304
USA
Tel +1 650 846 7500
Fax +1 650 845 6505
www.dupont.com

May 27, 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

GRN 000584

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 Cellulase enzyme preparation from *Penicillium funiculosum* 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

Cellulase enzyme preparation from *Penicillium funiculosum*

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

The cellulase is used as processing aid in brewing, baking 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

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 Cellulase Enzyme

Preparation Derived from

Penicillium funiculosum

Expressing the Cellulase Gene

From

Penicillium funiculosum

Is Generally Recognized As Safe

For Use in Food Processing

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

May 27, 2015

TABLE OF CONTENTS

1. GENERAL INTRODUCTION

The cellulase enzyme preparation under consideration is produced by submerged fermentation of a selected strain of *Penicillium funiculosum* producing its wild-type cellulase enzyme.

The enzyme product is intended for use as processing aid in the brewing, baking, and potable alcohol industries for the breakdown of the non-starch polysaccharides of grain and fungi to reduce viscosity. In these applications, the *P. funiculosum* PF8/403-M cellulase will primarily be replacing the cellulase from one of the other available commercial sources. In all of these applications, the cellulase 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 cellulases currently in use include cellulases derived from other microorganisms, most notably *Trichoderma reesei* and *Aspergillus niger*. These cellulases have been determined to be GRAS by DuPont Industrial Biosciences. In the case of cellulase enzyme from *P. funiculosum*, it was initially determined to be GRAS by DuPont Industrial Biosciences in 2004.

The accepted name of the principal enzyme activity is 1,4-(1,3;1,4)-β-D-glucan 4 glucanohydrolase. Other names used are endoglucanase, endo-1,4-beta-glucanase, carboxymethylcellulase, endo-1,4-beta-D-glucanase, beta-1,4-glucanase, beta-1,4-endoglucanhydrolase, celludextrinase, avicelase.

The enzyme catalyses the endohydrolysis of 1,4-β-D-glucosidic linkages in cellulose, lichenin and cereal β-D-glucans. It will also hydrolyse 1,4-linkages in β-D-glucans that also contain 1,3-linkages.

The EC number of the enzyme is 3.2.1.4 and the CAS number is 9012-54-8.

The information provided in the following sections is the basis of our determination of GRAS status of this cellulase 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 (*P. funiculosum*) for cellulase is discussed in Sections 2 and 7.

1.1 Exemption from Pre-market Approval

Pursuant to the regulatory and scientific procedures established in proposed 21 C.F.R. 170.36 (Appendix1), DuPont Industrial Biosciences has determined that its cellulase enzyme preparation produced by *Penicillium funiculosum* is a Generally Recognized as Safe ("GRAS") substance for

the intended food applications 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

Cellulase enzyme preparation from *Penicillium funiculosum*.

1.4 Applicable Conditions of Use

The cellulase is used as a processing aid in brewing, baking 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 MICROORGANISM

2.1 Production Strain

Penicillium funiculosum PF8/403-M is a derivative of a wild-type strain of *P. funiculosum*, IMI 134755, purchased from the International Mycological Institute (IMI). PF8/403-M was developed by Rhone-Poulenc and subsequently by Rhodia. The strain was directly derived from IMI 134755 by conventional mutagenesis methods. No foreign DNA was introduced into the strain during its development (Appendix 5).

PF8/SD403 is also referred to as SD101 as an IMI deposit for feed enzyme purposes and as IMI 378536 as an IMI deposit for patent purposes. Non-pathogenic and non-toxigenic *P. funiculosum* PF8/430-M has a long history of safe use in the production of industrial enzymes and chemicals of both food grade and technical grade.

The Joint FAO/WHO Expert Committee of Food Additives (JECFA) has evaluated the cellulase preparation from *P. funiculosum*. The material evaluated contains enzyme activities from cellulase, beta-glucanase, and xylanase with secondary enzyme activities from alpha-N-arabinofuranosidase, cellulose 1,4-ß-cellobiosidase, ß-glucosidase, and xylan 1,4-ß-xylosidase. The enzyme preparation is described as a product by the controlled fermentation of non-toxigenic and non-pathogenic strains of *P. funiculosum* and used in the preparation of fruit juices, wine, beer, and vegetable oils. No ADI was allocated, which is an indication that the material is unlikely to be hazardous. Cellulase enzyme preparations usually contain limited amounts of beta-glucanase and other hemicellulases. These components from various microbial sources also have been safely used in the food processing industry (Pariza and Johnson 2001).

2.2 Source Microorganism

The source organism is *Penicillium funiculosum* PF8/403-M (also called *Penicillium funiculosum* SD101). It is deposited in the International Mycological Institute strain collection as IMI 378536 under the Budapest Treaty, for patent purposes (patent application WO 99/57325). *P. funiculosum* is classified as a Biosafety Level 1 (BSL1) microorganism by the American Type Culture Collection (ATCC) based on assessment of the potential risk using U. S. Department of Public Health guidelines with assistance provided by ATCC scientific advisory committees, and is also considered as suitable for Good Industrial Large Scale Practice (GILSP) worldwide.

2.3 Stability of the modified strain

The production strain, modified by traditional means, is completely stable after industrial scale fermentation as judged by cellulase production using the production organism. The strain *Penicillium funiculosum* PF8/403-M is a stable strain, which can easily be maintained as a

homogeneous population under the usual laboratory and production conditions. The strain has been tested repeatedly for stability after growth for more than a dozen generations which occur in a large scale industrial fermentation and no significant instability was detected. By plating the strain on agar media, a low frequency of phenotypic and genotypic variants may be found. In this aspect the strain does not differ from other, highly specialized industrial micro-organisms.

2.4 Antibiotic activity / antibiotic resistance

The source organism does not produce antibiotics, and no antibiotic resistance genes were introduced in the production microorganism.

2.5 Absence of Production Microorganism in 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 the International Food Biotechnology Council (IFBC) is satisfactorily addressed.

3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1 Enzyme Identity

IUB Nomenclature:	1,4-(1,3;1,4)-ß-D-glucan 4-glucanohydrolase
IUB Number:	3.2.1.6
CAS Number:	62213-14-3
Reaction catalyzed:	Endohydrolysis of 1,4-ß-D-glucosidic linkages in cellulose, lichenin and cereal ß-D-glucans. It will also hydrolyze 1, 4-linkages in β-D-glucans that contain 1, 3-linkages.
Other Names:	Cellulase, endoglucanase, endo-1,4-beta-glucanase, carboxymethylcellulase, endo-1,4-beta-D-glucanase, beta-1,4-glucanase, beta-1,4-endoglucanhydrolase, celludextrinase, avicelase

3.2 Amino Acid Sequence

The amino acid sequence of the *P. funiculosum* PF8/403-M cellulase enzyme is shown in Appendix 2. *Penicillium funiculosum* PF8/403-M cellulase is similar to various other cellulases isolated from commercially relevant bacteria e.g., it is 73% homologous with the *Rasamsonia emersonii* (CBS 393.64) endo-beta-1,4-glucanase (GenBank KKA23484.1), beta-glucanase, cellulase and xylanase enzyme preparation derived from *Talaromyces emersonii* was affirmed by FDA as GRAS (GRN 479) and a cellulase enzyme preparation from *Myceliophthora thermophile* was also affirmed as GRAS (GRN 292).

4. MANUFACTURING PROCESS

This section describes the manufacturing process for the cellulase enzyme which follows standard industry practice (Kroschwits, 1994; Aunstrup *et al.*1979; Aunstrup, 1979). For a diagram of the manufacturing process, see Appendix 3.The quality management system used in the manufacturing process complies with the requirements of ISO 9001.The enzyme preparation is 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 cellulase concentrate are standard ingredients used in the enzyme industry (Kroschwits, 1994; Aunstrup *et al.*, 1979; Aunstrup, 1979). All the raw materials conform to the specifications of the Food Chemicals Codex (FCC), 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 Enzyme Technical Association (ETA) submission to FDA on antifoams and flocculants dated April 24, 1998 and the Agency's September 11, 2003 response to ETA. The maximum use level of the antifoam in the production process is $\leq$ 0.15%.

Glucose (produced from wheat) and soy flour will be used in the fermentation process which will be consumed by the microorganism as the nutrient. No other major allergen substances will be used in the fermentation and recovery processes.

4.2 Fermentation Process

The cellulase enzyme is manufactured by submerged fermentation of a selected, pure culture of the non-GM strain of *P. funiculosum* 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 *P. funiculosum* 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 appropriate. 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 sodium benzoate, the final formulation is:

Sodium benzoate	0.80-1.40%
Active enzyme protein	5-10%
Sorbitol	8.0-14.0%
Water and minor fermentation components add up to 100%	

Quality control of finished product

The final cellulase liquid concentrate from *P. funiculosum* is analyzed in accordance with the general specifications for enzyme preparations used in food processing as established by the Joint FAO/WHO Expert Committee on Food Additives (JEFCA) in 2006 and the Food Chemical Codex (FCC) 9th edition (U.S. Pharmacopeia, 2014). These specifications are described in Section 5.

5. COMPOSITION AND SPECIFICATIONS

5.1 Quantitative Composition

The cellulase enzyme product is formulated with sodium benzoate. The final formulation 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:	Min. 3150 CMC-DNS U/g
Sorbitol:	8.0-14% (w/w)
Cellulase:	5-10% (w/w)
Sodium Benzoate:	0.80-1.40% (w/w)
pH	3.7-4.2

Remainder is water (with minor % of fermentation components)

5.2 Specifications

The cellulase 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 Specifications (2006), including the absence of mycotoxins. Ochratoxin A is the only mycotoxin known to be produced by *P. verrucosum* however there is no evidence for the production of any mycotoxins by penicilli. DuPont Industrial Biosciences regularly tests the production for absence of mycotoxins to make sure that all test material is negative, according to Patterson and Roberts (1979) including Ochratoxin A. The results of analytical testing of the

three lots of the product is given in Appendix 4 verifying that it meets FCC (U.S. Pharmacopeia, 2014) and JECFA (2006) specifications for enzyme preparations.

6. APPLICATION

6.1 Mode of Action

Cellulase catalyzes the endohydrolysis of 1,4-β-D-glucosidic linkages in cellulose, lichenin and cereal β-D-glucans. It will also hydrolyze 1,4-linkages in β-D-glucans that contain 1,3-linkages. Such enzyme activity is widely present in nature and in particular in food ingredients. The substrates and the reaction products are themselves present in food ingredients. Consequently, no adverse effect on nutrients is expected.

6.2 Uses and Use Levels

In principle, the enzymatic conversion of cellulose, lichenin and cereal beta-D-glucans with the help of Cellulase may be used in the processing of all food raw materials which naturally contain cellulose, lichenin and cereal beta-D-glucans. The cellulase will be used as a processing aid in the brewing, baking and potable alcohol production.

6.2.1 Uses

Brewing:

Cellulase is typically added in mashing step to reduce the viscosity of the wort and improve the separation of the wort from the spent grains. Cellulase is therefore denatured already in the consecutive lautering or mash filtration step. Cellulase may also be added during the fermentation step. In this case Cellulase will be denatured during the pasteurization step. The cellulase also improves the final beer filtration. The main processes involved in brewing beer include:

- Milling: reducing the size of the dry malt
- Mashing: addition of water to the malt (the enzyme is added at this step)
- Lautering or mash filtration: removing the spent grains from the liquor (wort)
- boiling the mash with hops
- Fermentation
- Maturation, conditioning, beer filtration and packaging

High molecular-weight polysaccharides especially β-glucans in grain and malt have a significant impact on brewing process in that they increase mash viscosity and cause hazes and poor dewatering or removal of solids. Furthermore, the filterability of wort and beer is

poor. The malt contains some β-glucanase but the amount is variable and inadequate. The cellulase degrades barley β-glucans and arabinoxylans, which are extracted during the mashing process, thereby reducing the viscosity of the wort and improving the separation of the wort from the spent grains

Baking:

Cellulase aids in the conversion of cellulose and beta-D-glucans, especially for whole meal baking products. Cellulase typically performs its technological function during the dough or batter handling. Cellulase is denatured by heat during the baking or steaming step. The benefits of the use of Cellulase in baking may include:

- Improve handling of the dough (improved extensibility and stability)
- Improve dough structure and behaviour during the baking step
- Ensure a uniform and slightly increased volume and improve crumb structure of the bakery product, which might otherwise be impaired by industrial processing of the dough
- Reduce batter viscosity, beneficial in the production process for e.g. waffles, pancakes and biscuits
- Increase firmness and reduce oil absorption in instant noodles
- Reduce checking (formation of hair line cracks)
- Accelerate the drying step, thereby shortening the process time

Pasta and noodles production process:

In other baking related cereal based processes such as pasta and noodle production. Cellulase also performs its technological function during dough handling. In this case, Cellulase is denatured by heat during the drying, boiling or steaming step.

Potable alcohol production:

Cellulase has been used in Potable alcohol production for several decades. In potable alcohol production the high levels of cellulose, lichenin and beta-D-glucans results in high viscosity due to the water-binding capacity. High viscosity has negative effects on alcohol production because it limits solid concentration in mashing and reduces efficiency in the mixing, separation and filtration processes. Residual beta-D-glucans may also contribute to fouling in heat exchangers and distillation equipment.

Cellulase is added in the pre-treatment, liquefaction, pre-saccharification or the fermentation step. In potable alcohol production, solids are separated from the fermentation slurry at the end of fermentation and any enzyme protein precipitate will be removed with the solids. The liquids are distilled. The distilled alcohol is subsequently filtered through a molecular sieve at

temperatures well over boiling to adsorb further traces of water and water-soluble proteins. Therefore, the cellulase will not be present/active in the end product due to distillation in the case of alcohol production.

The benefits of the conversion of cellulose, lichenin and cereal beta-D-glucans with the help of a cellulase in Potable alcohol production are:

- Decrease viscosity
- Better processing (solid/liquid separation, resulting in higher solid concentration during mashing; increase fermentable sugars and improve mass transfer during fermentation)
- Reduce fouling in the heat exchangers and distilling equipment
- Increase flexibility in the choice of raw materials
- Potential higher alcohol yield as result of better processing, and thereby less use of raw materials.
- Reduce fuel consumption due to better heat transfer

6.2.2 Use Levels

Commercial food enzyme preparations are used by food manufacturers according to the *Quantum Satis* (QS) principle, i.e. at a level not higher than the necessary dosage to achieve the desired enzymatic reaction- according to the principles of current Good Manufacturing Practice (GMP). The table below shows the range of recommended use levels for each application where the food enzyme may be used.

Application	Raw material (RM)	Recommended use levels (mg TOS/kg RM)	Maximal recommended use levels (mg TOS/kg RM)
Baking	Flour	0.55-1.2	1.2
Brewing	Cereals	2.83-28.3	28.3
Potable alcohol production	Cereals	3.08-18.4	18.4

Doses are expressed in Total Organic Solids[1] (TOS).

[1] TOS is defined as Dry Matter minus ash. The amount of ash (e.g. mineral salts use in the fermentation) does generally not exceed a few percent.

6.3 Enzyme Residues in the Final Foods

DuPont Industrial Biosciences expects the cellulase to be inactivated or removed during the subsequent production processes for all applications. Cellulase activity is easily inactivated at temperature above 70°C and completely inactivated at 80°C. Therefore, in the brewing process, the enzyme will be inactivated during the heating of the mash. The mash off takes place at about

78°C for 15 minutes and then the temperature is raised to the boiling temperature (100°C) which is kept for about 60 minutes. We should not measure any enzyme activity in the final product. In the distilling process, the enzyme will be inactivated during the cooking step and removed during the distillation process. There is no enzyme protein in the final alcohol product.

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 (Pariza and Foster, 1983; Pariza & Johnson, 2001; and Pariza and Cook, 2010). 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. 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.' *Penicillium funiculosum* strains used in enzyme manufacture meet these criteria for non-toxigenicity and non-pathogenicity.

7.1.1 Safety of the host

Penicilli are universal fungi found almost everywhere on earth. The great majority of penicilli are saprophytes found in soil. The genus has long been regarded as benign, and of little or no consequence as human or animal pathogens. Human pathogenic species in this genus is limited to *P. marneffei* (*Samson et al.* 2000) and the infections typically occur in immuno-compromised individuals (LoBuglio and Taylor 1995). *Penicillium funiculosum* is a food-borne fungus that can be found commonly on tropical cereals, fruits, and nuts (Samson *et al.* 2000).There is no other evidence from the literature or experimental studies that associate *Penicillium funiculosum* with human pathogenicity.

Mycotoxins that currently are considered as adverse to human health include aflatoxins, ochratoxin A, fumonisins, certain trichothecenes, and zearalenone, judged by their potency and the degree of possible human exposure (Pitt 2000). Except for ochratoxin A, known to be produced by *P. verrucosum*, there is no evidence for the production of any of those mycotoxins by penicilli. DuPont Industrial Biosciences regularly tests the production for absence of mycotoxins to make sure that all test material is negative, according to Patterson and Roberts (1979) including Ochratoxin.

Penicillium is also known for its ability to produce antibiotics against fungi, bacteria, protozoa, viruses, and some tumour cells. However, a search of the literature indicates that only two strains of *P. funiculosum* have the capacity of producing ingredients with antibacterial (Singh *et al.* 1986) and anti-viral (Shope,1948) potential. Both of those ingredients have no clinical applications. Antibacterial activity was negative in the *P. funiculosum* cellulose producing strain PF8/408-M (Appendix 4).

A review of the literature search on the organism (May 2015) uncovered no reports that implicate *P. funiculosum* in any way with a disease situation, intoxication, or allergenicity among healthy adult humans and animals. The species is not present on the list of pathogens used by the EU (Directive Council Directive 90/679/EEC, as amended) and major culture collections worldwide. It is classified as a Biosafety Level 1 (BSL1) microorganism by the American Type Culture Collection (ATCC) based on assessment of the potential risk using U.S. Department of Public Health guidelines with assistance provided by ATCC scientific advisory committees. BSL1 microorganisms are not known to cause diseases in healthy adult humans.

The Joint FAO/WHO Expert Committee of Food Additives (JECFA) has evaluated the cellulase preparation from *P. funiculosum*. The material evaluated contains enzyme activities from cellulase, beta-glucanase, and xylanase with secondary enzyme activities from alpha-N-arabinofuranosidase, cellulose 1,4-ß-cellobiosidase, ß-glucosidase, and xylan 1,4-ß-xylosidase. The enzyme preparation is described as a product by the controlled fermentation of non-toxigenic and non-pathogenic strains of *P. funiculosum* and used in the preparation of fruit juices, wine, beer, and vegetable oils. No ADI was allocated, which is an indication that the material is unlikely to be hazardous. Non-pathogenic and non-toxigenic, *Penicillium funiculosum* PF8/403-M has been safely used for a long time as a production organism of food grade enzyme products for over 10 years. The cellulase conforms to the *Penicillium funiculosum* cellulase enzyme preparation evaluated by the JECFA and is used in the brewing and starch processing industries.

The cellulase by this specific production strain, PF8/403-M, has been determined to produce no adverse effects in the following toxicology tests: 1) Acute oral toxicity study in the rat, 2) Eye irritation study, 3) Inhalation study, 4) Skin irritation study, 5) Bacterial reverse mutation – Ames test, 6) *In vitro* mammalian chromosomal aberration test performed with human lymphocytes, and 7) 13-week oral (gavage) toxicity study in rats.

In addition, the food/feed grade products produced by *Pencillium funiculosum* to which this strain pertains were determined to be safe for their intended uses, and are Generally Recognized as Safe (GRAS).

From the information reviewed, it is concluded that the production organism *P. funiculosum* strain PF8/403-M provides no specific risks to human health and is safe to use as the production organism of cellulase. The strain is non-pathogenic and non-toxigenic.

7.2 Safety of the Manufacturing Process

The manufacturing process for the production of cellulase 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, resulting in a liquid cellulase enzyme preparation. The process is conducted in accordance with current food good manufacturing practice (cGMP) as set forth in 21 CFR Part 110. The resultant product meets the purity specifications for enzyme

preparations of the FCC, 9th Edition (US Pharmacopeia, 2014) and the general specifications for enzyme preparations used in food processing proposed by JECFA (2006). Although glucose from wheat and soy flour is used in the fermentation process, as they will be consumed by the microorganism as the nutrients, they will not pose any allergy risk in the final product.

7.3 Safety of *Penicillium funiculosum* Cellulase

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. Cellulase has been used in food process 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 enzymes residue in bread and other foods do not represent any unacceptable risk to consumers. Further, in a 2006 published 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, and despite that no novel sequence was introduced, the potential that this cellulase 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 *P. funiculosum* PF8/403-M is unlikely to pose a risk of food allergenicity.

The most current allergenicity assessment guidelines developed by the Codex Alimentarius 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 (Mirsky *et al.*, 2013).

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 *P. funiculosum* PF8/403-M cellulase expressed in *P.funiculosum* is given below in FASTA format, without its secretion signal.

QLHTSSRWILDANNNRVKLRCINWAGHIDLKIPEGLSKQPVDTITSWIADNGFNCVRLTYSIDMALDPTQSV
SDSFTAAGTAWNVESEMTDAYNAAVARNPFLAEASTLDVFAHVIDSLDNKGVMTILDNHVSRASWCCNL
TDGNGWWDTATGYIASNSRYFNTTEWLAGLDAMATFALDHPGVVGMSIRNELRPFPILQDVTHSDWYNY
VTQGALAVHNANPHVLVIIGGSQSATDLSFIKTSNLDFSQWAGKHVWEFHAYSFTVTFPGNTDCTVASAEY
GLLDGFLLTQNESYTAPLILSEFGVGQTGGPNSGFSDKDYNYLQCLVQYMESNDAEWIVWAVQGSYYIRD
GNVDYDETWGLLNHDWSDWRNSNFSSLLGKMWNVTQGP

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 no match to allergens by identity across 80 amino acids exceeding 35 %.

FASTA alignment of the sequence with known allergens revealed no match (using E-value <0.1 as the cut-off) to sequences in the data base using the full sequence search capabilities.

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.

In conclusion, based on the sequence homology alone, *Penicillium funiculosum* Cellulase is unlikely to pose a risk of food allergenicity.

As for all enzyme products, an MSDS for the cellulase 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.

7.3.2 Safety of use in food

In addition to the allergenicity assessment described above, the safety of this cellulase 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 new 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

DuPont Industrial Biosciences (Legacy Danisco) has conducted safety studies on cellulase derived from *P. funiculosum* PF8/403-M and contains all the enzyme components that are the subject of this GRAS notice. In addition, the cellulase was tested for antibacterial resistance using methods recommended by the JECFA (1991) and for aflatoxins, orchratoxin A, sterigmatocystin, T-2 toxin, and zearalenone. The enzyme product does not show any antibacterial resistance and none of the mycotoxins tested were detected.

For the purpose of toxicological tests, the enzyme complex of cellulase, beta-glucanase and hemicellulase from *Penicillium funiculosum* PF8/403-M was designated "*Penicillium funiculosum* concentrated liquid" for the 90-day study, and "Cellulase 2000L" for the other toxicological tests (acute oral toxicity, inhalation study, eye irritation study, skin irritation study, Bacterial reverse mutation assay, chromosome Aberration Assay).

The test materials used in all toxicology investigations have the following characteristics:

Characterization of Test material (*Penicillium funiculosum* concentrated liquid)

Batch Number:	TSL35/97
TOS (%)	8.37
Specific gravity:	1.032 g/ml
Enzyme activity:	2427 U/g solution
Total Organic Solids (TOS):	8.37%
Specific activity:	29924 U/g TOS

Characterization of Test Material (Cellulase 2000L)

Batch Number:	WE 540
Physical:	Non-viscous, clear light brown liquid
Specific gravity:	1.0278 g/ml
Enzyme activity, cellulase:	2042 U/g solution
Total Organic Solids (TOS):	5.74% (w/w)
Specific activity, cellulase:	35557 U/g TOS

The studies include:

A. 13-week Oral (Gavage) toxicity study in rats
B. 13-week Feeding study on *P. funiculosum* Cellulase CP (Strain 3094)
C. Acute Oral Toxicity
D. Inhalation
E. Eye irritation
F. Bacterial Reverse Mutation Assay
G. Chromosome aberration assay

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) A 13-week Oral (Gavage) Toxicity Study in Rats (SafePharm, No 854/004)

A 13-week oral toxicity test in rats was performed on the Cellulase sample designated "*Penicillium funiculosum* concentrated liquid" (Batch No. TSL35/97). The study was conducted in accordance with the methods recommended in OECD Guideline No. 408, "Repeated Dose 90-day Oral Toxicity Study in Rodents", September 1998.

a. Procedure
The objective of this study was to investigate the potential of a *Penicillium funiculosum* concentrated liquid to induce systemic toxicity after repeated daily oral administration. Groups of 10 Sprague Dawley Crl:CD®BR strain rats/sex each were gavaged daily with 0, 7.5, 37.5 or 75 mg total protein/kg body weight in a constant aqueous volume of 5 ml/kg body weight. These doses corresponded to, respectively, 0, 50, 250 or 1000 mg of test material/kg/day in a constant volume of 5 ml/kg. Control animals were treated with distilled water.

All animals were observed daily for mortality and signs of morbidity. Animals were housed in groups of up to four by sex in polypropylene grid floor cages suspended over trays lined with absorbent paper. The animals were allowed free access to food and water. Paper bedding, placed

beneath the cages, was changed at least 3 times per week. 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. 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.

b. Results
There were no treatment-related deaths in this study. There were no biological or statistical differences between the control and treated groups with respect to clinical observations, feed consumption, body weight, body weight gain, hematology, clinical chemistry and ophthalmologic examinations. The relative brain weight in high dose group females was marginally increased but was of questionable toxicological significance in the absence of histopathologic findings. There were no treatment-related changes in histopathology. All morphological changes were those commonly observed in laboratory rats of the age and strain employed.

c. Evaluation
Throughout the entire study, there were no treatment related effects in all parameters investigated, from clinical observations to histopathological examinations. The relative brain weight in high dose females was increased but of questionable significance in the absence of similar finding in males and associated histopathological finding.

Under the conditions of this assay, it can be concluded that oral gavage of *Penicillium funiculosum* concentrated liquid 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 1000 mg of *Penicillium funiculosum* concentrated liquid /kg body weight/day. This is equivalent to 83.7 mg TOS/kg bw/day.

2) Feeding Study with Cellulase CP from *Penicillium funiculosum* Strain 3094 (HRC, No. SRG 13/84237)

a. Procedure
The objective of this study was to investigate the potential of Cellulase CP (produced by *Penicillium funiculosum* 3094) to induce systemic toxicity after repeated daily oral administration. Groups of 15 CD rats/sex each were provided with a diet containing 0, 2000,

10000 or 50000 ppm of Cellulase CP. These doses corresponded to, respectively, 0, 50, 250 or 1000 mg/kg/day in a constant volume of 5 ml/kg. Control animals were treated with normal diet. All animals were observed daily for mortality and signs of morbidity. Animals were housed in groups of five in polypropylene grid floor cages suspended over trays lined with absorbent paper with fresh water *ad libitum*. Paper bedding, placed beneath the cages, was changed at least 3 times per week. All groups were housed under controlled temperature, humidity and lightning conditions. Body weight, water consumption 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 week 6 and at study termination. Hematology and clinical chemistry were conducted at week 6 and at study termination prior to necropsy, which was performed on all groups. After a thorough macroscopic examination, selected organs were removed, weighed and processed for future histopathological evaluation. Microscopic examination was conducted on selected organs from control and high dose animals and on the spleen of all males in all groups. 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.

b. Results

There were no treatment-related deaths in this study. There were no biological or statistical differences between the control and treated groups with respect to clinical observations, feed consumption, body weight, body weight gain, hematology, clinical chemistry and ophthalmologic examinations. During week 6, the RBC and WBC counts in high dose male animals were statistically different from control values (7.7×10^6/ mm^3 vs $7.9 \times 10^6/mm^3$ and $12.6 \times 10^3/mm^3$ vs. $15.7 \times 10^3/mm^3$, respectively). However, no differences were noted at study termination ($8.0 \times 10^6/mm^3$ vs. $8.0 \times 10^6/mm^3$ and 14.6×10^3 vs. $14.1 \times 10^3/mm^3$ for RBC and WBC, respectively). There were slight increases in spleen weights noted in treated males, being 0.73, 0.82, 0.84 and 0.76 g for the control, low, mid and high dose groups, respectively. However, these values were still within the historical control values for CD rats (0.70 – 0.90 g) and in the absence of similar effects in females, the findings were not considered as treatment-related. The blood glucose levels decreased in high dose females at weeks 6 and 12 and in high dose males at week 6 but all values were still within the historical control ranges for blood glucose in CD rats. No treatment-related changes in histopathology were found. All morphological changes were those commonly observed in laboratory rats of the age and strain employed.

c. Evaluation

Throughout the entire study, there were no treatment related effects in all parameters investigated, from clinical observations to histopathological examinations. Sporadic alterations in RBC and WBC were noted in high dose males at week 6 but were not considered as treatment related due to its transient effects.Variations in organ weights were noted but all values were still within the historical control values for CD rats. Based on the food intake, the concentration of Cellulase CP taken by the groups receiving 2000, 10000 and 50000 ppm was, respectively, 133, 666 and 3371 mg/kg/day for male rats and 151, 749, and 3836 mg/kg/day for females.

Under the conditions of this assay, it can be concluded that feeding of Cellulase CP in the diet for 90 continuous days did not result in systemic toxicity to rats. The NOAEL (No observed adverse effect level) is established at 50000 ppm (3371 mg/kg/day for males; 3836 mg/kg/day for females).

3) Acute oral toxicity study (HRC, No RNP 461a/950521/AC)

An acute oral toxicity study was performed on the Cellulase sample designated "Cellulase 2000L" (Batch No. WE 540). The study was conducted in accordance with the OECD guideline No. 401.

a. Procedure
The acute oral toxicity of cellulase was investigated in the rat. A limit dose of 2000 mg/kg was given to a group of 5 male and 5 female Sprague Dawley rats. All animals were observed for mortality, morbidity and signs of toxicity over a period of 14 days. All animals were killed on day 15 and a necropsy performed.

b. Results
There were no deaths and no overt signs of toxicity were noted throughout the entire investigation period. All animals achieved satisfactory bodyweight gains throughout the study and no abnormalities were recorded at the macroscopic examination on Day 15.

c. Evaluation
Based on the oral LD50 of greater than 2000 mg/kg, cellulase is classified as "practically non-toxic" by ingestion.

4) Inhalation study (HLS, No. RNP 522/971453)

An inhalation study was performed on the cellulase sample designated "Cellulase 2000L" (Batch No. WE 540). The study was conducted in accordance with the OECD guideline No. 303.

a. Procedure
An acute inhalation limit test was conducted with *Penicillium funiculosum* concentrated powder. Five male and five females were exposed to 2.25 mg/L of the test material for 4 hours. This was the highest attainable concentration. All animals were observed for mortality, morbidity, and behavioral changes for 14 days.

b. Results
The mass median aerodynamic diameter was 4.3 μm, which was slightly above the ideal range of 1 to 4 μm. Approximately 76% of the particles were in the respirable range of < 7 μm. There were no deaths and no overt signs of toxicity relative to clinical observations, body weight, food consumption and gross observations.

c. Evaluation
Based on the LC_{50} value of > 2.25 mg/L (highest attainable concentration), the concentrated powder is classified as "slightly toxic" by inhalation. Since the concentration of enzyme in the concentrated powder is approximately 15 fold higher than cellulase, it can be expected that cellulase would be "practically non-toxic" by inhalation.

5) Eye irritation study (HRC, No. RNP 463a/950569/SE)

An eye irritation study was performed on the Cellulase sample designated "Cellulase 2000L" (Batch No. WE 540). The study was conducted in accordance with the OECD guideline No. 405.

a. Procedure
Primary eye irritation studies were conducted with three New Zealand rabbits. A 0.1 ml of the test material (equivalent to 209.8 units of enzyme activity) was instilled onto the conjunctival sac of one eye of each rabbit and ocular irritation was evaluated at 1, 24, 48, and 72 hours post instillation and at day 4 and 7 post instillation. Untreated eye served as control.

b. Results
No corneal damage or iritis was observed. Only a mild conjunctival irritation was noted in all 3 animals at the 1-hour observation but all cleared at subsequent observations. The PIS was 2.0/110.0

c. Evaluation
Under the conditions of this investigation, cellulase is classified as slightly irritating.

6) Skin irritation study (HRC, No. RNP 462a/950547/SE)

A skin irritation study was performed on the Cellulase sample designated "Cellulase 2000L" (Batch No. WE 540). The study was conducted in accordance with the OECD guideline No. 404.

a. Procedure
Acute dermal irritation studies were conducted in New Zealand rabbits. The test material was applied as a single dose of 0.5 ml (equivalent to 1049 units of enzyme activity) on one 6 cm^2 intact site/rabbit and kept in contact for 4 hours. At the end of the exposure period, the skin was washed and dermal irritation was evaluated at 1, 24, 48 and 72 hours following patch removal.

b. Results
No dermal reactions were noted and the Irritation Score was 0.0/8.0

c. Evaluation
Under the conditions of these experiments, cellulase is not a skin irritant.

7) Bacterial reverse mutation assay – Ames Assay (HRC, No RNP 460a/950320)

An Ames test was performed on the cellulase sample designated "Cellulase 2000L" (Batch No. WE 540). The study was conducted in accordance with the OECD guideline No. 471.

a. Procedure
The objective of this assay is to assess the potential of cellulase to induce point mutation (frame-shift and base-pair) in five strains of *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 mix). The tests were performed using the plate incorporation method, at 5 dose levels, in triplicates. The doses selected for the assay were based on the enzyme activity of the test material (2041 U/ml). A preliminary toxicology test was carried out with dose levels ranging from 0.2 to 204.1 U/plate.

In the main assay, five concentrations of the test material were assayed in triplicates against each tester strain in both the presence and absence of metabolic activation. The test material (0.1 ml) was nixed with 0.1 ml of bacterial suspension, 2 ml of histidine deficient agar, 0.5 ml of S-9 mix or 0.5 ml 0.1M phosphate buffer (pH 7.4) and overlaying into sterile plates of agar. Sterile distilled water served as vehicle control. The positive controls used for assays without S-9 mix were N-ethyl-N'-nitro-N-nitrosoguanidine, 2-nitrofluorene, and 9-aminoacridine and the positive control used for assays with S-9 mix was 2-aminoanthracene. After 72 hours incubation at 37°C, the plates were assessed for the number of revertant colonies. The main assay was repeated twice.

b. Results
In the preliminary assay, cellulase was not toxic to the test bacteria up to and including the highest dose level tested (204.1 U/plate) (maximum achievable concentration based on the concentration of the test article) in both the presence and absence of S-9 mix. The test material caused no visible reduction in the growth of the bacterial background lawn at any dose level. Therefore, 204.1 U/plate was selected as the highest dose level for the main tests.
In the main tests, five dose levels (2.04, 6.12, 10.41, 61.24 and 204.1 U/plate) were used. Neither precipitate nor appreciable cytotoxicity was observed. No biologically or statistically significant increases in the number of revertant colonies were observed in any dose level in either main test. 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, cellulase has not shown any evidence of mutagenic activity in the Ames assay in both presence and absence of metabolic activation.

8) *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes (HRC, No RNP 459/951239)

An *in vitro* mammalian cytogenicity test was performed with the cellulase sample designated Cellulase 2000L (Batch No. WE 540). The test was conducted under GLP and in accordance with the guideline recommended by OECD "*In Vitro* Mammalian Chromosome Aberration Test", No. 473.

a. Procedure
The objective of this assay is to investigate the potential of cellulase to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). Cellulase 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 preliminary assay, all cultures containing S9 were treated for 3 hours and the cell pellets re-suspended in fresh medium. They were then incubated for an additional 15 hours. Cultures without S9 were incubated for 18 hours. After the incubation period, the culture tubes were centrifuged, the supernatant was aspirated and the cells were re-suspended in fresh medium. Two hours prior to the scheduled cell harvest, Colcemid 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, the proportion of mitotic cells per 1000 cells was recorded. From these results, a dose level causing a decrease in mitotic index of 50% was selected as the highest dose in the confirmatory assay.

The preliminary toxicity assay was conducted with concentrations of cellulase ranging from 0.05 to 5.86 U/ml. The confirmatory test was conducted with cultures incubated for 18 and 32 hours. The dose levels of cellulase used ranged from 0.09 to 5.86 U/ml in the absence of S9 and 0.18 to 5.86 U/ml in the presence of S9.

b. Results
In the preliminary dose-range assay, cytotoxicity (58% reduction in mitotic index) was observed at the 5.86 U/ml dose level in the absence of S9. At a dose level of 2.93 U/ml, the MI was reduced to 61% of the control level, and was chosen as the highest dose in the confirmatory assay. In the confirmatory assay, Cellulase did not cause any statistically significant increase in chromosomal aberrations in this mammalian system. The percentage of cells with structural or numerical aberrations was not significantly increased in all cellulase-treated cultures.

c. Evaluation
No biologically or statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with cellulase both in the presence and absence of metabolic activation (S-9 mix). Under the conditions of this test, there is no evidence to suggest that cellulase induces chromosomal aberrations (both structural and numerical) in mammalian cells both in the presence and absence of metabolic activation.

CONCLUSION

In summary, cellulase is practically non-toxic by ingestion and by inhalation. It is not an irritant to the skin and slightly irritating to the eyes. In genotoxicity studies using bacterial or mammalian systems, cellulase is not a mutagen or clastogen in both the presence and absence of metabolic activation. This summary also includes a 90-day feeding study on Cellulase CP, a cellulase preparation derived from *P. funiculosum* 3094, a strain from which PF8/403-M was developed (Appendix 5). This feeding study further supports our safety assessment on cellulase enzyme preparations derived from this *P. funiculosum* lineage.

Daily administration of cellulase by gavage for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at the highest dose tested of 1000 mg of *Penicillium funiculosum* concentrated liquid /kg body weight/day. This is equivalent to 83.7 mg TOS/kg bw/day. Also, daily administration of cellulase CP in a 13-week feeding study did not result in overt signs of systemic toxicity. The NOAEL is established at 50000 ppm (3371 mg/kg bw/day for males and 3836 mg/kg bw/day for females.

7.4 Overall Safety Assessment and Human Exposure

7.4.1 Identification of the NOAEL

In the 90-day oral (gavage) study in CD rats, a NOAEL was established at the highest dose tested of 1000 mg total protein/kg body weight/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 cellulase is through oral ingestion, selection of this NOAEL is thus appropriate.

NOAEL = 1000 mg/kg bw/day
= 83.7 mg TOS/kg bw/day

7.4.2 Human Exposure to Cellulase

Cellulase from *Penicillium funiculosum* may be used in the manufacture of a wide variety of foods, food ingredients and beverages. Due to this wide variety of applications, the most appropriate way to estimate the human consumption in the case of food enzymes is using the so-called Budget Method (Hansen, 1966; Douglass *et al.*, 1997). This method enables to calculate a Theoretical Maximum Daily Intake (TMDI) based on conservative assumptions regarding physiological requirements for energy from food and the energy density of food rather than on food consumption survey data.

The Budget Method was originally developed for determining food additive use limits and is known to result in conservative estimations of the daily intake; it is also based on the following

assumed consumption of important foodstuffs and beverages (for less important foodstuffs, e.g. snacks, lower consumption levels are assumed):

Average consumption over the course of a lifetime/kg body weight/day	Total solid food (kg)	Total non-milk beverages (l)	Processed food (50% of total solid food) (kg)	Soft drinks (25% of total beverages) (l)
	0.025	0.1	0.0125	0.025

In addition to the assumptions from the Budget Method, it is assumed that beer is consumed in the same amount as soft drinks.

EXPOSURE ASSESSMENT

In section 6.2.2, the recommended use levels of the enzyme cellulase are given, based on the raw materials used in the various food processes. For the calculation of the TMDI, the maximum use levels are chosen. Furthermore, the calculation takes into account how much food or beverage is obtained per kg raw material and it is assumed that all the TOS will end up in the final product. Per the table below, the application rate of Cellulase used in potable alcohol (distilled spirits) is 18.4 mg TOS/ kg of Raw Material (RM). In the case of alcohol distillation, the TOS will likely not be found in the final product.

Application		Raw material (RM)	Maximal recommended use level (mg TOS/kg RM)	Example Final food (FF)	Ratio RM/FF	Maximal level in FF (mg TOS/kg food)
Beverages	Brewing*	Cereals	28.3	Beer	0.17	4.8
	Potable Alcohol*	Cereals	18.4	Distilled Spirits	2.83	52
Solid	Baking	Flour	1.2	Bread, etc.	0.71	0.85

*It takes approximately 17 kg grist such (e.g. malted barley) to make 100 litres of finished beer.
The estimated yield from 1 m3 grist (mash) is 35% or 350 L of potable alcohol.

However, for the purpose of selecting an overall maximum exposure via liquids, the worst-case TOS concentration in beer (28.3 mg TOS/kg RM) is appropriate, because:

- The application rate of the cellulase enzyme to raw materials in brewing and cereal beverage is higher than that for potable alcohol (distilled spirits).
- Taking the respective process yields into account, the resulting worst-case exposure in beer is higher (on an equal alcohol content basis) than in either cereal beverages or 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 cellulase.

The Total TMDI can be calculated on basis of the **maximal** values found in food and beverage (in the above cases Brewing and baking) that could theoretically be carried-over to final foods and drinks, and multiplied by the average consumption of food and beverage/kg body weight/day. Consequently, the Total TMDI will be:

TMDI in food (mg TOS/kg body weight/day)	TMDI in beverage (mg TOS/kg body weight/day)	Total TMDI (mg TOS/kg body weight/day)
0.85x0.0125=0.011	4.8x0.025=0.12	**0.131**

It should be stressed that this Total TMDI is based on conservative assumptions and represents the worst case scenario because of the following reasons:

- It is assumed that all producers of the above mentioned foodstuffs and beverages use the specific enzyme Cellulase from *Penicillium funiculosum*.
- It is assumed that all producers apply the highest use level per application;
- For the calculation of the TMDI's in foods and beverages, those foods and beverages containing the highest theoretical amount of TOS were selected as representative for total foods and beverages.
- It is assumed that no TOS is removed in the food production process.
- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed daily over the course of a lifetime;

- Assumptions regarding food and beverage intake of the general population are overestimates of the actual average levels (Douglass *et al.*, 1997).

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 Total Theoretical Maximal Daily Intake (TMDI). As shown above, the Total TMDI of the food enzyme is 0.131 mg TOS/kg body weight/day. Consequently the Margin of Safety (MoS) is:

MoS = 83.7 mg TOS/kg body weight/day / 0.131 mg TOS/kg bw/day

= 639

As evident by the calculation, the Total TMDI is exaggerated. Moreover, the NOAEL was based on the highest dose administered, and is therefore to be considered as a minimum value. Therefore, the actual MoS in practice will be higher.

7.4.3 Conclusion

The safety of cellulase, as a food processing aid in brewing, baking and potable alcohol production, is assessed in a battery of toxicology studies investigating its genotoxicity and systemic toxicity potential.

Summarizing the results obtained from the several toxicity studies the following conclusions can be drawn:

- No mutagenic or clastogenic activity under the given test conditions was observed;
- The sub-chronic oral toxicity study showed a No Observed Adverse Effect Level (NOAEL) of at least 83.7 mg TOS/kg body weight/day.

The Margin of Safety (MoS) for human consumption can be calculated by dividing the NOAEL by the Total Theoretical Maximal Daily Intake (TMDI). The Total TMDI of the food enzyme is 0.131 mg TOS/kg body weight/day.

Consequently, the MoS is:

MoS = 83.7 mg TOS/kg body weight/day / 0.131 mg TOS/kg bw/day

= 639

The Total TMDI is exaggerated. Moreover, the NOAEL was based on the highest dose administered, and is therefore to be considered as a minimum value. Therefore, the actual MoS

in practice will be higher. The proposed application rates of cellulase are not a health concern and are supported by existing toxicology data.

8. BASIS FOR GENERAL RECOGNITION OF SAFETY

The cellulase derived from *Penicillium funiculosum* PF8/403-M is 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' own unpublished safety studies, support such a conclusion. *P. funiculosum* 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 cellulase enzyme preparation was determined to be acceptable for the proposed uses. For verification whether the NOAEL is sufficient to support a 100- fold safety margin in the intended uses, the safety studies conducted on the cellulase produced by the production strain described in this submission established a NOAEL at greater than 1000 mg concentrated liquid/kg body weight/ day or 83.7 mg TOS/kg bw/day.

Based on a worst-case scenario, the use of cellulase in brewing and manufacture of potable alcohol and bakery application is not expected to result in adverse effects to humans. A safety margin of 639 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 cellulase from *Penicillium funiculosum* strain PF8/403-M is safe and suitable for use in in the brewing, distilling, fiber and carbohydrate processing, as well as in feed applications. 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 – 21CFR 170.36

Appendix 2 – Amino acid sequence of *P. funiculosum* PF8/403-M

Appendix 3 – *P. funiculosum* PF8/403-M Cellulase production process

Appendix 4 – Certificates of Analysis

Appendix 5 – *Penicillium funiculosum* Strain lineage and summary of safety studies

Appendix 6 – Analysis of Safety based on Pariza and Johnson Decision Tree

Appendix 7 – GRAS concurrence letter from Dr. Pariza

10. LIST OF REFERENCES

Aunstrup, K., Andersen, O., Falch, E. A., and Nielsen, T. K. (1979) Production of Microbial Enzymes in Microbial Technology, 2nd ed.,Volume 1. Eds. Peppler, H.J., and Perlman, D., Chapter 9, pp. 282-309.

Aunstrup, K. (1979) Production, Isolation, and Economics of Extracellular Enzymes in Applied Biochemistry and Bioengineering, Volume 2, Enzyme Technology, Eds. Wingard, L.B., Katchalski-Katzir, E. and Golsdstein, L. pp. 28-68.

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

Bindslev-Jensen C., P.S. Skov, E.L. Roggen, P. Hvass, D.S. Brinch. (2006) Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry. *Food Chem. Toxicol.* 44: 1909-1915.

Codex Alimentarius Commission. (2003) ALINORM 03/34: Joint FAO/WHO Food Standard Programme, Codex Alimentarius Commission, 25th Session, Rome, Italy 30 June-5 July, Appendices III and IV, 2003, pp. 47–60.

Codex Alimentarius Commission. (2009) Joint FAO/WHO Food Standard Programme, Codex Alimentarius Commission, Rome, Italy.

Douglass, J. S., Barraj, L. M., Tennant, D. R., Long, W. R., & Chaisson, C. F. (1997) Evaluation of the budget method for screening food additive intakes. *Food Additives & Contaminants*, *14*(8), 791-802.

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

FAO. (2001) Evaluation of the Allergenicity of Genetically Modified Foods: Report of a Joint FAO/WHO Expert Consultation, Rome 2001.

FDA. (1999). Direct food substances affirmed as generally recognized as safe: cellulase enzyme preparation derived from *Trichoderma longibrachiatum* for use in processing food. Fed. Reg. 64:28358-28362.

GRAS Notice 479. Beta-glucanase, cellulase, and xylanase enzyme preparation from *Talaromyces emersonii.* http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=479

GRAS Notice 292. Cellulase enzyme preparation derived from a genetically modified strain of *Myceliophthora thermophile.*

http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=292

Greenough, R. J.; Everett, D. J. and Stavnsbjerg, M. S. (1991) Safety evaluation of alkaline cellulase. *Food Chem. Toxic.* 29:781-785.

Herman, R. A., Song, P., & ThirumalaiswamySekhar, A. (2009) Value of eight-amino-acid matches in predicting the allergenicity status of proteins: an empirical bioinformatic investigation. *Clin. Mol. Allergy*, *7*: 9.

Hansen, S. C. (1966). Acceptable daily intake of food additives and ceiling on levels of use. *Food Cosmet. Toxicol.,* 4:427-432/

Hjortkjaer, R.K., Bille-Hansen, V., Hazelden, K.P., McConville, M., McGregor, D.B., Cuthbert, J.A., Greenough, R.J., Chapman, E., Gardner, J.R. and Ashby, R. (1986) Safety evaluation of Celluclast®, an acid cellulase derived from *Trichoderma reesei*. *Journal of Food and Chemical Toxicology.* 24(1): 55-63.

Huntingdon Life Sciences (HLS) (1997). *Penicillium funiculosum* concentrated powder – acute (four-hour) inhalation study in rats, Report No. RNP 522/971453. (unpublished)

Huntingdon Research Center (HRC) (1995). Cellulase 2000L - Acute oral toxicity to the rat, Report No. RNP 461a/950521/AC. (unpublished)

Huntingdon Research Center (HRC) (1995). Cellulase 2000L - Eye irritation to the rabbit, Report No. RNP 463a/950569/SE. (unpublished)

Huntingdon Research Center (HRC) (1995). Cellulase 2000L - Skin irritation to the rabbit, Report No. RNP 462a/950547/SE. (unpublished)

Huntingdon Research Center (HRC) (1995). Cellulase 2000L - Bacterial Mutation Assay, Report No. RNP 460a/950320. (unpublished)

Huntingdon Research Center (HRC) (1995). Cellulase 2000L - Metaphase chromosome analysis of human lymphocytes cultured *in vitro*, Report No. RNP 459/951239. (unpublished)

Huntingdon Research Center (HRC) (1985). Cellulase CP - Toxicity to rats by continuous dietary administration for 13 weeks, Study No. SRG 13/84237. (unpublished)

IFBC (International Food Biotechnology Council). (1990) Chapter 4: Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms in Biotechnologies and Food: Assuring the Safety of Foods Produced by Genetic Modification. *Regul. Toxicol. Pharmacol.*12: S1-S196.

JECFA (Joint FAO/WHO Expert Committee on Food Additives). (2006) General specifications and considerations for enzyme preparations used in food processing. In: Compendium of food

additive specifications, volume 4. Analytical methods, test procedures and laboratory solutions used by and referenced in the food additive specifications. pp. xxi-xxv. FAO/JECFA Monographs 1. Rome.

Kroschwitz, J.I. (1994) Enzyme Applications in Encyclopedia of Chemical Technology, 4th edition.Volume 9, pp. 567-620.

Ladics, GS, RF Cressman, C Herouet-Guicheney, RA Herman, L Privalle, P Song, JM Ward, S McLain. (2011) Bioinformatics and the allergy assessment of agricultural biotechnology products: Industry practices and recommendations. *Regul. Toxicol. Pharmacol.* 60: 46-53.

LoBuglio, K. F. and Taylor, J. W. (1995) Phylogeny and PCR identification of the human pathogenic fungus *Penicillium marneffei*. *J. Clin. Microbiol.* 33:85-89.

Merget, R.; Sander, I.; Raulf-Heimsoth, M. and Baur, X. (2001) Baker's asthma due to xylanase and cellulase without sensitization to alpha-amylase and only weak sensitization to flour. *Int. Arch. Allergy Immunol.* 124:502-505.

Metcalfe DD, Astwood JD, Townsend R, Sampson HA *et al.,* (1996) Crit. Rev. *Food Sci. Nutr.* 36S: 165 –186.

Mirsky, H.P., Cressman, R.F., and Ladics, G.S. (2013). Comparative assessment of multiple criteria for the *in silico* prediction of allergenic cross-reactivity. *Reg. Toxicol. Pharmacol.*, 67:232-239.

O'Connor, T. M.; Bourke, J. F.; Jones, M. and Brennan, N. (2001) Report of occupational asthma due to phytase and beta-glucanase. *Occup. Environ. Med.* 58:417-419.

OECD (Organization for Economic Cooperation and Development). (1993) Safety Evaluation of Foods Derived by Modern Biotechnology.

Pariza, M.W., and M Cook. (2010) Determining the safety of enzymes used in animal feed. *Regul Toxicol Pharmacol.* 56:332-342.

Pariza, M. W. and E. A. Johnson. (2001) Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing – Update for a New Century. *Regul. Toxicol. Pharmacol.* 33: 173-186.

Pariza, M.W. and Foster, E. M. (1983) Determining the Safety of Enzymes Used in Food Processing. *J. Food Prot.*, 46: 453-468.

Patterson DS, Roberts BA. (1979) Mycotoxins in animal feedstuffs: sensitive thin layer chromatographic detection of aflatoxin, ochratoxin A, sterigmatocystin, zearalenone, and T-2 toxin. *J Assoc Off Anal Chem.* Nov; 62(6):1265–1267

Pitt, J. I. (2000) Toxigenic fungi: which are important? *Med. Mycol.* 38 (Suppl. 1):17-22.

Quirce, S.; Cuevas, M.; Diez-Gomez, M.; Fernandez-Rivas, M.; Hinojosa, M.; Gonzalez, R. and Losada, E. (1991) Respiratory allergy to *Aspergillus*-derived enzymes in bakers' asthma. *J. Allergy Clin. Immunol.* 90:970-978.

SafePharm Laboratories Ltd. (1998), *Penicillium funiculosum* concentrated liquid: 90-day repeated oral (gavage) toxicity in the rat. Report No. SPL 854/004. (unpublished)

Samson, R. A.; Hoekstra, E. S.; Frisvad, J. C. and Filtenborg, O. (2000) Introduction to food-and airborne fungi. 6th Ed. CBS: The Netherlands.

Shope, R. E. (1948) Therapeutic activity of a substance from *Penicillium funiculosum* Thom against swine influenza virus infection of mice. *Am. J. Bot.* 35:803.

Singh, P. D.; Johnson, J. H.; Aklonis, C. A. and O'Sullivan, J. (1986) SQ 30,957, a new antibiotic produced by *Penicillium funiculosum*. Taxonomy, fermentation, isolation, structure determination, synthesis and antibacterial activity. *J. Antibiot.* (Tyoko) 39:1054-1058.

US Pharmacopeia.(2014) Monograph: Enzyme Preparations. In: Food Chemicals Codex, 9th *Edition*. The United States Pharmacopeia Convention, Rockville, Maryland, pp. 410-415.

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 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/locations/

(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.

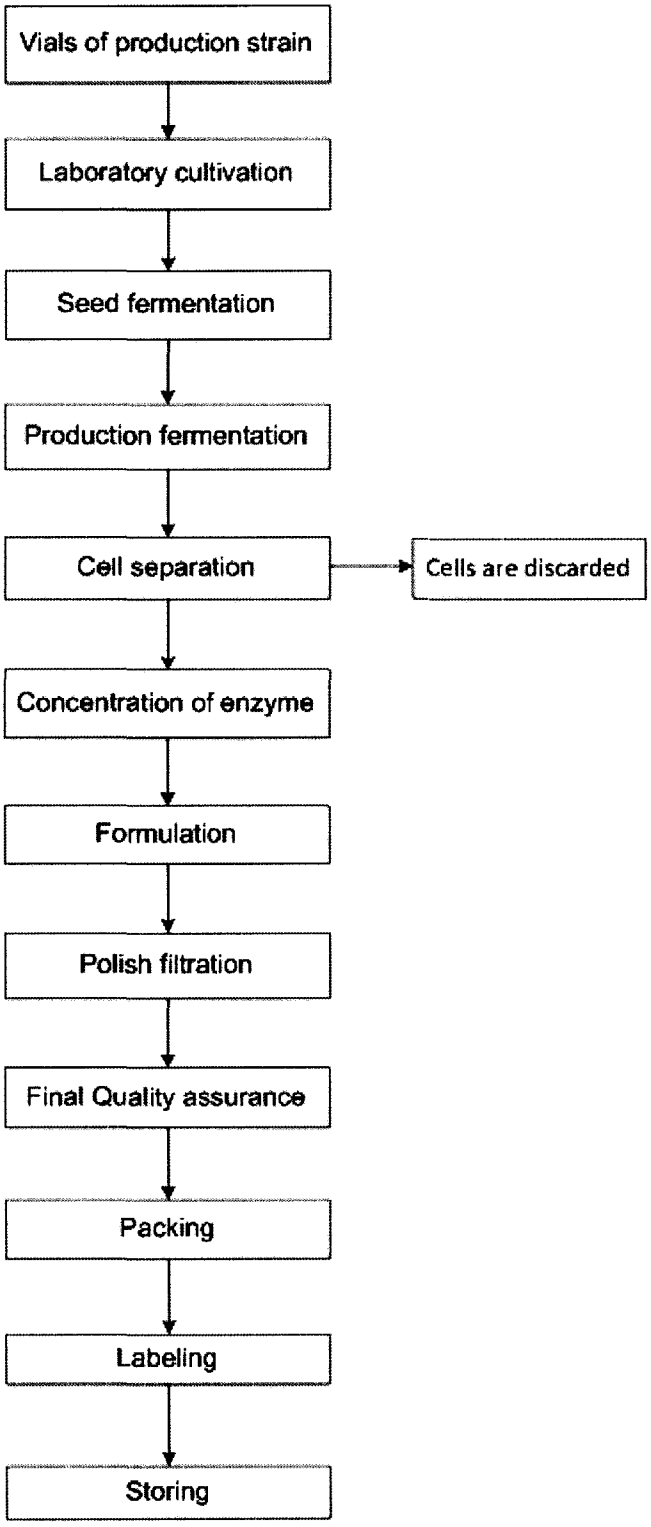
(l) New information may at any time require reconsideration of the GRAS status of a food ingredient. Any change in part 182, part 184, or Sec. 186.1 of this chapter shall be accomplished pursuant to Sec. 170.38.

[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 *P. funiculosum* PF8/403-M cellulase

QLHTSSRWILDANNNRVKLRCINWAGHIDLKIPEGLSKQPVDTITSWIADNGFNCVRLTYSIDMALDPTQSV
SDSFTAAGTAWNVESEMTDAYNAAVARNPFLAEASTLDVFAHVIDSLDNKGVMTILDNHVSRASWCCNL
TDGNGWWDTATGYIASNSRYFNTTEWLAGLDAMATFALDHPGVVGMSIRNELRPFPILQDVTHSDWYNY
VTQGALAVHNANPHVLVIIGGSQSATDLSFIKTSNLDFSQWAGKHVWEFHAYSFTVTFPGNTDCTVASAEY
GLLDGFLLTQNESYTAPLILSEFGVGQTGGPNSGFSDKDYNYLQCLVQYMESNDAEWIVWAVQGSYYIRD
GNVDYDETWGLLNHDWSDWRNSNFSSLLGKMWNVTQGP

Appendix 3: *P. funiculosum* PF8/403-M Cellulase production process



Appendix 4- CERTIFICATES OF ANALYSIS

P. funiculosum PF8/408-M cellulase (Cellulase 2000L)

Genencor International

Genencor International Inc.
200 Meridian Centre Blvd.
Rochester, N.Y. 14618 - 3916
U.S.A.
+1.800.847.5311
+1.585.244.4544 fax

Genencor International B.V.
P.O.Box 218
2300 AE Leiden
The Netherlands
+31.71.5686.168
+31.71.5686.169 fax

Genencor International Asia Pacific Pte Ltd
61 Science Park road
The Galen #06- 16 East Wing
Singapore Science Park III
Singapore 117525
+65.6511.5600
+65.6511.5666 fax

Genencor International Argentina, S.A.
1750 Alicia Moreau De Justo St
Capital Federal
Buenos Aires, Argentina
+54.11.5199.9550
+54.11.51999559 fax

Product Specification

Date Revised 21- JAN- 2008

Product Name: **Cellulase 2000L**
Item Code: A03180

Assay	Unit	Low Spec	High Spec
ENZYME ACTIVITIES			
Cellulase	CMC- DNS U/g	3150	
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/ml	0	50000
Total Coliforms	CFU/ml	0	30
E. coli	/25ml		NEG
Salmonella	/25ml		NEG
Production Strain	/ml		NEG
Antibacterial Activity	/ml		NEG
PHYSICAL PROPERTIES			
pH		3.7	4.2
Appearance			Clear, light brown liquid
OTHER ASSAYS			
Heavy Metals as Pb	mg/kg	0	30
Arsenic	mg/kg	0	3
Cadmium	mg/kg	0.00	0.50
Mercury	mg/kg	0.00	0.50
Lead	mg/kg	0	5
Mycotoxins			NEG

This product complies with the current recommended purity specifications for food- grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC).

***Penicillum funiculosum* concentrated powder (CPD30)**

Name

Penicillum funiculosum concentrated powder (CPD30) (intermediate in the manufacture of ROVABIO™ BETA-GLUCANASE PF P and ROVABIO™ XYLAN P).

Chemical description

Cellulase (EC 3.2.1.4; CAS no. 9012-54-8; EINECS no. 232-734-4) produced by the fermentation of a deposited strain of *Penicillium funiculosum*. Whilst this preparation contains a range of different enzyme activities including endo-1,3(4)-β-glucanase (EC 3.2.1.6) and endo-1,4-β-xylanase (EC 3.2.1.8) it is characterized by assay for cellulase activity.

Physical description

A powder enzyme preparation

Batch

TSL 5/97
manufactured 24 January 1997

Purity

Activity = 31247 u/g (DNS CMC method)

Other activities= 22216 u/g (DNS barley β-glucan method)
= 8204 u/g (DNS birchwood xylan method)
= 87191u/g (viscometric wheat arabinoxylan method)

free from the viable production microorganisms	
total viable count	260 $cfu.g^{-1}$
yeasts and moulds	< 1 $cfu.g^{-1}$
coliforms	< 1 $cfu.g^{-1}$
Escherichia coli	absent in 25 g
Salmonella	absent in 25 g

Appendix 5 – *Penicillium funiculosum* Strain lineage and summary of safety studies

3086 ≡ *Penicillium funiculosum* IMI 134755

This is included for completeness only. *Penicillium funiculosum* IMI 134755 is the wild type and was mutated, by Selby *et al.* by UV irradiation.

↓

3087 ≡ ***Penicillium funiculosum* IMI 134756 (starting point)**

(a) *heat treatment* ↓

WG6/24

(1) *UV irradiation* ↓

WG28/16

(2) *UV irradiation* ↓

WG31/11

(3) *β-irradiation* ↓

PF2/150 ≡ **PF3094**

(5) *UV irradiation* ↓

WG51/14

(7) *UV irradiation* ↓

WG54/6

(9) *purification* ↓

WG54/6/29

(10) *UV irradiation* ↓

PF8/403-M ≡ ***Penicillium funiculosum* PF8/403-M, SD101**

Toxicology Test Summaries *P. funiculosum* PF8/408-M cellulase

PRODUCTION ORGANISM	ENZYME PREPARATION	TOXICOLOGY TEST	RESULT
P. funiculosum **(PF8/403-M)**	**Cellulase** (Cellulase 2000L)	Acute Oral Toxicity Study	No signs of toxicity at LD_{50} of greater than 2000 mg/kg
		Inhalation Study	Non-toxic
		Eye irritation Study	Non-irritant
		Skin irritation Study	Non-irritant
		Bacterial Reverse Mutation Assay-Ames Test	Non-mutagenic
		In vitro Mammalian Chromosomal Aberration Test performed with Human Lymphocytes	Non-clastogenic
	Cellulase (concentrated liquid)	13-Week Oral (gavage) Toxicity Study in Rats	No adverse effects. NOEL established at greater than 1000 mg of *P. funiculosum* concentrated liquid/kg body weight/day or 83.7 mg TOS/kg bw/day.
P. funiculosum **(3094)**	**Cellulase**	13-Week Feeding Study	No adverse effects. NOAEL established at 50000 ppm, 3371 mg/kg/day for males and 3836 mg/kg/day for females.

Appendix 6: Pariza & Johnson Decision Tree on *P. funiculosum* PF8/408-M expressed in *P. funiculosum*

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

Yes. *Go to 2.*

2. Is the production strain modified using rDNA techniques?

No. *Go to 5.*

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

7. Is the production strain non-pathogenic?

Yes. *Go to 8.*

8. Is the Test Article free of antibiotics?

Yes. *Go to 9.*

9. Is the Test Article free of oral toxins known for members of this species?

Yes. *Go to 11.*

11. Is the NOAEL for the test substance sufficiently high to ensure safety?

Yes. Oral administration of *Penicillium funiculosum* cellulase concentrated liquid, to rats for a period of 90 consecutive days at dose levels of up to 1000 mg.Kg^{-1}(bodyweight) day^{-1} produced no toxicologically significant changes in the parameters measured. The "No Observed Effect Level" (NOEL) was, therefore, considered to be 1000 mg Kg^{-1}(bodyweight) day^{-1}. Additionally, in a separate, similar 90-day feeding study using *P. funiculosum* strain 3094 cellulase CP, no adverse effects occurred, and the NOAEL was established at 50000 ppm (3371 mg/kg/day for males and 3836 mg/kg/day for females.

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

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

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

Although, the production strain was not well-characterized, the production strain does come from a safe strain lineage as presented in Appendix 5. Its safety as a production host and methods of modifications are well documented, and their safety has been confirmed through repeated toxicology testing (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 *Penicillium funiculosum* PF8/403-M cellulase expressed in *Penicillium funiculosum* is safe and suitable for use as a processing aid in the brewing, baking and potable alcohol production and is Generally Recognized as Safe (GRAS) for those uses.

Appendix 7- GRAS Concurrence letter from Dr. Michael W. Pariza

Michael W. Pariza Consulting LLC
7102 Valhalla Trail
Madison, WI 53719

Michael W. Pariza, Member

November 13, 2004

Cynthia Blumenthal
Specialist, Regulatory Affairs
Genencor International, Inc.
925 Page Mill Road
Palo Alto, CA 94304

Dear Ms. Blumenthal,

I have reviewed the information you provided on Genencor International's cellulase enzyme preparations derived from a non-pathogenic and non-toxigenic strain of *Penicillium funiculosum.*, The enzymes are designated designated Cellulase 2000L (produced using *P. funiculosum* PF8/403) and Cellulase CP (produced using *P. funiculosum* 3094). In this evaluation I considered the biology and safe lineage of the production organisms, the long safe history of use of cellulase enzyme preparations in food, the manufacturing method, and safety data generated by Genencor.

The parental lineage leading to the development of *P. funiculosum* 3094 and PF8/403 began with a non-pathogenic and non-toxigenic strain of *P. funiculosum* designated IMI 134755, which was subsequently modified by traditional UV irradiation-induced mutation. *P. funiculosum* PF8/403 has been safely used in food enzyme manufacture for more than 10 years. The toxicological evaluation of Cellulase 2000L and PF8/403 involved a standard battery of *in vitro* and *in vivo* tests including 90-day subchronic feeding challenges in rats.

Based on this information I concur with the evaluation made by Genencor, that Cellulase 2000L and Cellulase CP are safe for use as a

processing aid at minimum levels necessary to accomplish the intended technical effects in accordance with Current Good Manufacturing Practices (CGMPs) to catalyze the breakdown of non-starch polysaccharides in grain and fungi used in the brewing, wine and potable ethanol industries.

Please note that this is a professional opinion directed at safety considerations and not an endorsement, warranty, or recommendation regarding the possible use of Cellulase 2000L or Cellulase CP by you or others.

Sincerely,

(b) (6)

Michael W. Pariza
Wisconsin Distinguished Professor
Member, Michael W. Pariza Consulting LLC

SUBMISSION END