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ORIGINAL SUBMISSION

#567

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GRN 000567

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OFFICE OF
FOOD ADDITIVE SAFETY

January 26, 2015

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

RE: GRAS Notification - Exemption Claim

Dear Dr. Gaynor,

Pursuant to the proposed 21C.F.R. § 170.36 (c) (l) Danisco US Inc. (operating as DuPont Industrial Biosciences) hereby claims that Xylanase enzyme preparation produced by *Trichoderma reesei* expressing the gene encoding xylanase from *Aspergillus niger* 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

Xylanase enzyme preparation from *Trichoderma reesei* expressing the gene encoding the xylanase X3 from *Aspergillus niger* (Xylanase X3).

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

The xylanase is used as a processing aid in brewing, potable alcohol, cereal beverage manufacture, and bakery application

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

This GRAS determination is based upon scientific procedures.

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

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

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

Sincerely,

(b) (6)

(b) (6)

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

Enclosures (3 binders)

A Xylanase Enzyme Preparation Derived from

Trichoderma reesei

Expressing the Xylanase Gene

from

Aspergillus niger

Is Generally Recognized As Safe

For Use in Food Processing

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

January 26, 2015

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1. GENERAL INTRODUCTION

The xylanase enzyme preparation under consideration is produced by submerged fermentation of *Trichoderma reesei* carrying the native *xln3* gene from *Aspergillus niger (tubingensis)* encoding the wild-type xylanase enzyme (hereafter named as Xylanase X3).

The enzyme product is intended for use as processing aid in brewing, potable alcohol, cereal beverage manufacture, and bakery application. In these applications, Xylanase X3 will primarily be replacing the xylanase from one of the other available commercial sources. In all of these applications, Xylanase X3 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 xylanases currently in use include xylanases derived from other microorganisms, most notably *Bacillus subtilis and T. reesei,* but also from *A. niger*. These xylanases have been determined to be GRAS by DuPont Industrial Biosciences. Xylanase X3 was determined to be GRAS by DuPont Industrial Biosciences in 2013.

The accepted name of the principle enzyme activity is 1,4-β-D-xylan xylanohydrolase. Other names used are endoxylanase, endo-(1,4)-beta-xylanase, 1,4-beta-D-xylan xylanohydrolase , 1,4-beta-xylanase, beta-D-xylanase, beta-endoxylanase and beta-xylanase.

The enzyme catalyses endohydrolysis of (1->4)-beta-D-xylosidic linkages in xylans (various 1,4-β-D-xylooligosaccharides).

The EC number of the enzyme is 3.2.1.8, and the CAS number is 9025-57-4.

The information provided in the following sections is the basis of our determination of GRAS status of this Xylanase X3 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 (*T. reesei*) for this Xylanase X3 is discussed in Sections 2 and 7. Another essential aspect of the safety evaluation of enzymes derived from genetically modified microorganisms is the identification and characterization of the inserted genetic material (Pariza & Johnson, 2001; IFBC, 1990; EU SCF, 1991; OECD, 1993; Berkowitz and Maryanski, 1989). The genetic modifications used to construct this production organism are well defined and are described in Section 2. The safety evaluation described in Section 7 shows no evidence to indicate that any of the transferred DNA codes for or express a harmful toxic substance.

1.1 Exemption from Pre-market Approval

Pursuant to the regulatory and scientific procedures established in proposed 21 C.F.R. 170.36 (Appendix 1), DuPont Industrial Biosciences has determined that its xylanase enzyme preparation produced by *Trichoderma reesei* expressing the gene encoding xylanase X3 from *A. niger* 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

Xylanase enzyme preparation from *Trichoderma reesei* expressing the gene encoding the xylanase from *A. niger* (xylanase X3).

1.4 Applicable Conditions of Use

The xylanase is used as a processing aid in brewing, potable alcohol, cereal beverage manufacture, and bakery application.

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

The production organism is a strain of *Trichoderma reesei*, which has been genetically modified by inactivation of several cellulase genes, and by over expression of the native *A. niger* xylanase gene for the production and secretion of Xylanase X3 enzyme. *T. reesei* 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. It also meets the criteria for a safe production microorganism as described by Pariza and Johnson (2001). One expression cassette was used to introduce the *A. niger* gene encoding Xylanase X3 under the regulation of the native *T. reesei cbhI* (cellobiohydrolase 1) promoter and terminator. The expression cassette used the endogenous *T. reesei* orotate phosphoribosyl transferase (*pyr2*) gene as a selectable marker. In addition the strain also contains a cassette with a variant of the endogenous *T. reesei* acetolactate synthase (*als*) gene to replace previously integrated wild type TrGA cassettes. This mutant *als* gene confers chlorimuron ethyl (herbicide) resistance. The expression cassettes were integrated into the recipient chromosome.

2.2 Host Microorganism

The host microorganism *T. reesei* strain RL-P37 was obtained from Dr. Montenecourt. The derivation and characterization of strain RL-P37 has been published by Sheir-Neiss and Montenecourt (1984). Strain RL-P37 is a cellulase over-producing strain that was obtained through several classical mutagenesis steps from the wild-type *T. reesei* strain (QM6a). Strain QM6a is present in several public culture collections, e.g. in the American Type Culture Collection as ATCC 13631. *T. reesei* has more recently been identified as a clonal derivative or anamorph of *Hypocrea jecorina* (Dugan, 1998).

2.3 Donor Microorganism

The donor strain used as a source for the Xylanase X3 gene was *A. niger* 10M-61. 10M-61 was obtained from the wild type *A. niger* from the DuPont (legacy Danisco) strain collection after UV mutagenesis and selection for improved xylanase activity.

A. niger has a long history of safe use in the production of industrial enzymes and chemicals of both food grade and technical grade. The species is listed as a production/donor organism for a series of food-grade carbohydrases, oxidoreductases, lipases, glucanotransferase, and proteases in the Pariza and Johnson paper (2001). In addition, several *A. niger* derived substances used in the food industry have acquired GRAS status. Alpha-amylase, glucoamylase, various pectinase

enzymes, aspartic proteinase, glucose oxidase, and catalase were the subjects of GRAS Notice 89. In 1997, *A. niger* became one of the ten microbial species/strains that were eligible for exemption under 40 CFR Part 725 as recipient microorganisms under the TSCA biotechnology regulation (US EPA, 1997), based on EPA's risk assessment. Also, the safety of *A. niger* was reviewed in great detail by Schuster *et al.* (2002) and was also discussed by Olempska-Beer *et al.* (2006).

2.4 Expression Cassettes

Two expression cassettes were used. The Xylanase X3 expression cassette contained the *T. reesei cbh1* promoter and terminator, genomic *xln3* DNA encoding the *A. niger* xylanase, and the endogenous *T. reesei* orotate phosphoribosyl transferase (*pyr2*) gene, as a selectable marker for transformation of *T. reesei.* The *T. reesei* acetolactate synthase (*als*) gene was used as a selectable marker in a cassette for deletion of the wild type glucoamylase gene. The final production strain is designed as Brew2#21.

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

2.5 Stability of the Introduced Genetic Sequences

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

2.6 Antibiotic Resistance Gene

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

2.7 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

IUBMB Nomenclature[1]:	1,4-β-D-xylan xylanohydrolase
IUBMB Number:	3.2.1.8
CAS Number:	9025-57-4
Reaction catalyzed:	Hydrolysis of terminal 1,4-linked α-D-glucose residues successively from non-reducing ends of the chains with release of β-D-glucose.
Other Names:	Endohydrolysis of (1→4)-β-D-xylosidic linkages in xylans (various 1,4-β-D-xylooligosaccharides)

3.2 Amino Acid Sequence

The amino acid sequence of the *Aspergillus niger* xylanase (Xylanase X3) enzyme is known and is shown in Appendix 2. The sequence of mature xylanase X3 protein is similar to various other xylanases isolated from commercially relevant fungi, e.g., it is 94% homologous with *Aspergillus kawachii* xylanase and 81% homologous to xylanase of *Rhizopus. oryzae*.

4. MANUFACTURING PROCESS

This section describes the manufacturing process for the xylanase 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 Xylanase X3 concentrate are standard ingredients used in the enzyme industry (Kroschwits, 1994; Aunstrup *et al.*, 1979;

[1] IUBMB. Enzyme nomenclature. Recommendations 1992 http://www.chem.qmw.ac.uk/iubmb/enzyme; last updated October 14, 2014.

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 $\leq 0.15\%$.

4.2 Fermentation Process

Xylanase X3 enzyme is manufactured by submerged fermentation of a selected, pure culture of the genetically modified strain of *T. reesei* 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 *T. reesei* 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 ~40% glycerol, ~1.4% sodium citrate, ~0.6% citric acid, ~0.2 % sodium benzoate, ~0.1% potassium sorbate, at pH 4.8. The remaining is water.

Quality control of finished product

The final xylanase liquid concentrate from *T. reesei* 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 liquid concentrate is stabilized with the formulation ingredients listed below and tested to demonstrate that it meets the specifications.

Various commercial formulations exist, with a range of enzyme activities. The following is a representative composition:

Enzyme activity:	129,500 XBU/g
Glycerol	~40.0%
Na-citrate	~1.40%
Citric Acid	~0.60 %
Sodium benzoate:	~0.20 %
K-sorbate	~0.10 %
pH:	4.8

5.2 Specifications

Xylanase X3 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 T2-toxin, the only mycotoxin of concern for this species.

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

As noted above, Xylanase X3 causes endohydrolysis of (1→4)-β-D-xylosidic linkages in xylans (various 1,4-β-D-xylooligosaccharides). The main reaction products of the Xylanase X3 will be hydrolysation products of xylan like i.e. xylo-oligosaccharides, xylose and arabinose.

6.2 Use and Use Levels

6.2.1 Uses

Xylanase X3 will be used as a processing aid where the enzyme is not present in the final food or present as inactive residue in negligible amounts.

Brewing and Potable Alcohol:

In brewing, Xylanase X3 is used to modify the grist xylan reducing wort and beer viscosity, improving filtration processes, and increasing yield. It will be used in the mashing of malted cereal, unmalted cereal and other plant sources including barley, corn, wheat, rye, milo, rice, tapioca and potatoes. After mashing, the wort is separated from the spent grains via filtration and ultimately boiled for 1-1.5 hrs for sterilization. With a temperature of 100 °C during this process the enzyme product will be completely inactivated. The resultant process liquors (worts) are fermented, typically by yeast, to produce ethanol.

In potable alcohol, Xylanase X3 is used to degrade xylan from malted cereal, cereal and other plant sources (includes barley, corn, wheat, rye, milo, rice, tapioca and potatoes). This will result in a decrease in mash viscosity, better processing, reduction in fuel consumption due to better

heat transfer and a potential increase in alcohol yield. The resultant process liquors (worts) are fermented, typically by yeast, to produce ethanol (and sometimes organic acids).

Cereal beverage:

Xylanase X3 will be used in cereal beverage production processes to hydrolyze xylan, hence supporting production by decreasing viscosity and increasing yield. The cereal beverage process is more or less similar to brewing.

Bakery:

Xylanase X3 will be used in various baking applications. The enzyme will be primarily used in yeast raised bread products and tortilla. The use of the enzyme provides improved baking performance of flour such as 1) volume increase of baked goods, 2) improved dough stability, 3) increased tolerance towards variations in process parameters, and 4) improved dough handling. In bakery applications the xylanase is added either to the flour or when mixing the dough. The xylanase is used as a processing aid supporting dough stability and handling and will be inactivated during the baking process.

6.2.2 Use Levels

Brewing
:
In brewing application the maximum use level for Xylanase X3 is 22.5 mg protein per kg grist (e.g. malted barley) and approximately 17 kg of grist are used to make 1 hectoliter (100 liters) of finished beer.

Potable Alcohol:

In potable alcohol manufacture the maximum use level for Xylanase X3 is 7.5 mg protein per kg grist and the approximate alcohol yield from grain is 40 %.

Cereal beverage:

In cereal beverage production (sorghum, oat, barley) the maximum use level for Xylanase X3 is 3.75 mg protein per kg grist.

Bakery:

In bakery applications, the maximum inclusion rate is 22.5 mg protein per kg flour.

6.3 Enzyme Residues in the Final Foods

DuPont Industrial Biosciences expects Xylanase X3 to be inactivated or removed during the subsequent production processes for all applications.

In brewing and cereal beverage production, the enzyme product is added in mashing, where it hydrolyzes the beta-glucans of the mash (mixture of milled gelatinized malt, gelatinized adjunct, and water) reducing wort viscosity. After mashing, the wort is separated from the spent grains via filtration and ultimately boiled for 1-1.5 hrs for sterilization. With a temperature of 100 °C during this process, the enzyme product will be completely inactivated.

In potable alcohol applications, Xylanase X3 is typically added in the mashing step. Upon conclusion of the fermentation, solids are separated from the fermentation slurry and any Xylanase X3 protein precipitate will be removed with the solids. This is estimated to be at least 50% of the protein. The liquids are then distilled with the temperature at the bottom of the still at approximately180°F (82 °C). Not only is the Xylanase X3 protein irreversibly denatured at this temperature, given the poor solubility of enzyme protein in alcohol, virtually none of it (<1%) will be carried in the evaporated alcohol. The distilled alcohol is subsequently filtered through a molecular sieve at temperatures well over boiling (149 °C) to adsorb further traces of water (and along with it, any water soluble protein), during which process the Xylanase X3 enzyme is denatured and any residuals are effectively removed from the alcohol such that no protein, including Xylanase X3, remains in the final product.

In bakery applications the Xylanase X3 is added either to the flour or when mixing the dough, and will be inactivated during the baking process.

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.' *T. reesei* meets these criteria for non-toxigenicity and non-pathogenicity.

7.1.1 Safety of the host

T. reesei was first isolated from nature in 1944. The original isolate, QM 6a, and its subsequent derivatives have been the subject of intense research due to their usefulness in the production of cellulases. In the 1980s, it was suggested by Bissett (1984) that *T. reesei* be placed into synonymy with *T. longibrachiatum*. Later however, evidence emerged indicating that the two species are not identical (Meyer *et al.,* 1992). The proposal by Kuhls *et al.* (1996) that *T. reesei* was a clonal derivative of *Hypocrea jecorina* is being accepted by more and more people in the science community, and the US National Center for Biotechnology Information (NCBI) refers to *T. reesei* as the anamorph of *H. jecorina* and no longer includes it in the genus *Trichoderma*. Therefore, the names *Trichoderma reesei*, *Trichoderma longibrachiatum,* and *Hypocrea jecorina* may appear in different regulatory approval documents and positive lists but they refer to essentially the same microorganism species.

A review of the literature search on the organism (December 2014) uncovered no reports that implicate *T. reesei* 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.

Brückner and Graf (1983) reported the isolation from *T. reesei* strain QM 9414 a peptaibol compound (paracelsin) that exhibited antibiotic activity. Their work was confirmed by another group that found evidence of peptaibol production in two other *T. reesei* strains (Solfrizzo *et al.*, 1994). However, peptaibols' antibiotic activity is clinically useless and commercially irrelevant, and the growth conditions under which the compounds were produced are very different from those in enzyme manufacturing. The US EPA published a risk assessment (Federal Register /Vol. 77, No. 172 / September 5, 2012 / pages 54499-54511) to support tiered exemption status for *T. reesei* QM6A and its derivates (including QM9414), in which the Agency acknowledged that under normal submerged fermentation conditions paracelsin is not produced. Strain QM 9414 and its derivatives have been safe producers of commercial cellulase enzyme preparations for food applications. The industrial enzyme preparations are still confirmed by the enzyme manufacturers not to have antibiotic activity according to the specifications recommended by JECFA (2006).

T. reesei has a long history of safe use in industrial scale enzyme production. The safety of this species as an industrial enzyme producer has been reviewed by Nevalainen et al. (1994) and Blumenthal (2004). The organism is considered non-pathogenic for humans and does not produce fungal toxins or antibiotics under conditions used for enzyme production. It is generally considered a safe production organism and is the source organism of a range of enzyme products that are used as processing aids in the international food and feed industries. It is listed as a safe production organism for cellulases in the Pariza and Johnson (2001) paper and Olempska-Beer *et*

al. (2006). Various strains have been approved to produce commercial enzyme products internationally, for example, in Canada (Food and Drugs Act Division 16, Table V), the United States (21CFR § 184.1250), Mexico, Brazil, France, Denmark, Australia/New Zealand, China, and Japan. To date, seven enzymes produced in *T. reesei* have been notified to FDA/CFSAN as GRAS for their intended uses (http://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&sort=GRN_No&order=DESC&startrow=1&type=basic&search=reesei).

The production organism of the xylanase enzyme preparation, the subject of this submission, is *T. reesei* strain Brew2#21. The strain has been genetically modified by inactivation of several cellulase genes and by overexpression of the native *A. niger* Xylanase X3 gene for the production and secretion of *T. reesei* xylanase enzyme. The strain was derived from RL-P37, a commercial production strain, which was derived from several classical mutagenesis steps, from the well-known wild-type strain QM6a. Virtually all strains used all over the world for industrial cellulase production today are derived from QM6a. DuPont Industrial Biosciences (formerly Genencor, a Danisco Division) has used strain RL-P37 for production of cellulases for over fifteen years and has developed many production strains from it using recombinant DNA techniques.

The xylanase by this specific production strain, Brew2#21, has been determined to produce no adverse effects in the following toxicology tests: 1) acute dermal irritation study in rabbits, 2) acute eye irritation/corrosion study in rabbits 3) bacterial reverse mutation – Ames assay, 4) *In vitro* mammalian chromosomal aberration test performed with human lymphocytes, and 5) 13-week oral (gavage) toxicity study in rats. In addition, all of the food/feed grade products produced by the lineage to which this strain pertains were determined to be safe for their intended uses, and are the subject of numerous GRAS determinations. GRAS Notices have been filed for four of the enzyme products from this strain lineage: chymosin, transglucosidase, acid fungal protease and glucoamylase, and FDA has issued "no questions" letters in response to each (see GRAS Notice 230, 315, 333, 372 respectively).

From the information reviewed, it is concluded that the production organism *T. reesei* strain Brew2#21 provides no specific risks to human health and is safe to use as the production organism of Xylanase X3. The strain is non-pathogenic and non-toxigenic.

7.1.2 Safety of the donor source

The donor strain used as a source for the Xylanase X3 gene was *A. niger (tubingensis)* 10M-61. The xylanase gene was not modified during construction of the subject microorganism, Brew2#21.

A. niger has a long history of safe use in the production of industrial enzymes and chemicals of both food grade and technical grade. The species is listed as a production/donor organism for a series of food-grade carbohydrases, oxidoreductases, lipases, glucanotransferase, and proteases in Pariza and Johnson (2001).

In addition, several *A. niger* derived substances used in the food industry have acquired GRAS status. Alpha-amylase, glucoamylase, various pectinase enzymes, aspartic proteinase, glucose oxidase, and catalase were the subjects of GRAS Notice 89. *A. niger* pectate lyase produce in *T. reesei* was notified as GRAS in GRN 32. In 1997, *A. niger* became one of the ten microbial species/strains that were eligible for exemption under 40 CFR Part 725 as recipient microorganisms under the TSCA biotechnology regulations (US EPA, 1997), based on EPA's human health and environmental risk assessments.

Also, the safety of *A. niger* was reviewed in great detail by Schuster *et al.* (2002) and was discussed by Olempska-Beer *et al.* (2006). Both concluded that *A. niger* is a safe source organism provided that good manufacturing practices are followed, including testing for applicable mycotoxins.

DuPont Industrial Biosciences has performed toxicological evaluation of ultrafiltration concentrate of xylanase preparation from the classical strain *A. niger* 10M-61. The conclusion is that xylanase preparation from *A. niger* is nonhazardous based on acute oral study. A battery of genotoxicity assays was conducted and under the conditions of these assays xylanase is not toxic, not a mutagen, a clastogen, or an aneugen.

As such, the safety of the donor organism is only minimally relevant, since only one isolated chromosomal DNA fragment encoding the Xylanase X3 enzyme was obtained from the donor organism, and used in the final production organism.

7.2 Safety of the Manufacturing Process

The manufacturing process for the production of Xylanase X3 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 xylanase 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).

7.3 Safety of *Trichoderma reesei* Xylanase

7.3.1 Allergenicity

According to Pariza and Foster(Pariza and Foster (1983)), there have been no confirmed reports of allergies in consumers caused by enzymes used in food processing.

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, the potential that Xylanase X3 could be a food allergen was assessed by comparison with sequences of known allergens and is summarized here. The *A. niger* Xylanase X3 mature peptide sequence was subjected to FASTA alignment of the sequence with known allergens using the peer-reviewed FARRP database, version 14 (Jan 2014) containing 1706 sequences. (http://www.allergenonline.org/index.shtml)

Guidelines developed by FAO/WHO (2001) as discussed and modified by the Codex Alimentarius Commission (2003) and republished in 2009 recommend the use of FASTA or BLASTP search for matches of 35% identity or more over 80 amino acids; these guidelines further state that a search for stepwise contiguous identical amino acid segment searches may be performed to identify sequences that may represent linear epitopes, but that these techniques need to be scientifically validated. This cautionary statement relates to the fact that ILSI/IFBC had previously advocated the use of matches of at least 8 contiguous identical amino acids (Metcalfe et al., 1996) but that the 2001 Joint FAO/WHO Expert Consultation (FAO,2001) suggested use 6-8 identical amino acid segments in contiguous searches. Following the FAO (2001) guidelines but taking the comments in Codex (2003, 2009) into account, two searches against the FARRP database were performed.

A primary search, using a sliding window of 80-amino acids and greater than 35% identity to indicate a match revealed no matches for *A. niger* Xylanase X3.

Although cautioned against in Codex (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.

It is concluded that *A. niger* Xylanase X3 is unlikely to pose a risk as a food allergen.

Like any other protein foreign to the human body, *A. niger* Xylanase X3 may cause respiratory sensitization. However, with the proper engineering controls, product formulation and handling instructions (avoiding dust or aerosols) this can effectively be prevented.

7.3.2 Safety of use in food

In addition to the allergenicity assessment described above, the safety of Xylanase X3 has also been established using the Pariza and Johnson (2001) decision tree, see Appendix 5. The Pariza and Johnson evaluation resulted in the conclusion to accept the enzyme preparation as safe without the need for new toxicology testing based on the history of safe use of xylanase and being produced by a production strain pertaining to a well-documented Safe Strain Lineage. Notwithstanding this initial conclusion, the safety of the enzyme preparation was further confirmed through unpublished toxicological testing as described below. The toxicology testing was conducted to satisfy regulatory requirements in countries where toxicology testing on the subject enzyme is required for enzyme preparation approval.

7.3.3 Safety Studies

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.

DuPont Industrial Biosciences (Legacy Danisco) has conducted two safety studies on *T. reesei* itself, one on a recombinant strain derived from RLP-37 used to make endoglucanase 1, and another on strain A83 used to make cellulase, xylanase and beta-glucanase, also derived from RLP-37 through mutation and selection. DuPont Industrial Biosciences has also conducted many safety studies on *T. reesei* derived enzymes (see Appendix 6).

In addition, DuPont Industrial Biosciences recently conducted five studies on the *A. niger* Xylanase X3 enzyme produced from *T. reesei* Brew2#21 the subject of this safety assessment. The test material contained 182.29 mg total protein/ml and 15.25% Total Organic Solids (TOS) Summaries are included below. In addition, the analytical testing of the tox lot was given in Appendix 7.

A. Acute dermal irritation study in rabbits

a. Procedure:
The objective of this study was to assess the local irritant effect of Xylanase X3. This study was conducted according to the method recommended in the OECD Guideline No. 404, April 2002.

This study was conducted in a stepwise manner. Initially, 5/10 of a milliliter of the test substance was applied to the skin of one rabbit for 4 hours. Since no dermal irritation was noted in this rabbit, a confirmatory test was completed with two additional healthy rabbits.

Approximately 24 hours prior to application, all animals were prepared by clipping the dorsal area and the trunk. Five tenth of one milliliter was applied to one 6 cm^2 intact dose site and covered with 1 inch x 1 inch, 4-ply gauze pad. The pad and entire trunk of each animal were then wrapped with semi-occlusive tape. Elizabethan collars were placed on each rabbit. After 4 hours

of exposure, the pads and collars were removed and the test sites were cleansed of any residual test substance. The grade of skin reaction was scored according to the Draize's scoring system immediately following patch removal and at 30-60 minutes, 24, 48 and 72 hours after patch removal.

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

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

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

B. **Acute eye irritation/corrosion study in rabbits**

a. Procedure
The objective of this study was to assess the ocular irritation potential of Xylanase X3. This study was conducted according to the method recommended in the OECD Guideline No. 405, 24 April 2002 and evaluated according to the Commission Directive 2001/59/EC of 6 August 2001.

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

b. Results
No ocular irritation was noted in all three rabbits.

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

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

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

a. Procedure:
The objective of this assay was to assess the potential of Xylanase X3 to induce point mutations (frame-shift and base-pair) in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and *Escherischia coli* strain WP2 uvrA. The test material was tested both in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). The assay was performed in two phases using the plate incorporation methodology for the positive control, 2-aminoanthracene, with *E. coli* and the treat and plate methodology for the all remaining strains and assays.

A screening (dose range) test was performed first to select the dose levels for the confirmatory assay. Vehicle control, positive control and 8 doses of the test article were plated, two plates per dose, with overnight cultures of all four strains of *Salmonella typhimurium* and *E. coli* WP2 *uvr*A in the presence and absence of S-9 mix. In the confirmatory assay, 6 doses of the test article along with appropriate vehicle and positive controls were plated in triplicate in the presence and absence of S-9 mix. All dose levels were expressed in terms of total protein (TP). The highest dose level tested was 5000 µg TP/plate, which is the maximum dose required by the OECD guideline. The positive controls used for assays without S-9 mix were 2-nitrofluorene, N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and ICR-191. For assays with S-9 mix, the positive control was 2-aminoanthracene. Vehicle control plates were treated by the addition of sterile deionized water.

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

b. Results:
In the screening assay, Xylanase X3 was not toxic to the test bacteria up to and including the highest dose level tested (5000 µg TP/plate) in both the absence and presence of S-9 mix. No positive mutagenic responses were observed with any of the tester strains in the presence of S-9mix or with tester strains TA1535, TA1537 and WP2 *uvr*A in the absence of S-9 mix. Based on the findings of the screening assay, 5000 µg TP/plate was selected as the highest dose level for the confirmatory assay.

In the confirmatory assay, six dose levels (50, 150, 500, 1500, and 5000 µg TP/plate) were tested. Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of metabolic activation.

Statistical increases in the number of revertant colonies were noted with the positive controls in both the presence and absence of metabolic activation substantiating the sensitivity of the treat and plate assay and the efficacy of the metabolic activation mixture.

c. Evaluation
Under the conditions of this assay, Xylanase X3 has not shown any evidence of mutagenic activity in the Ames assay in both presence and absence of metabolic activation.

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

a. Procedure

The objective of this assay was to investigate the potential of Xylanase X3 to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). In this assay, human lymphocytes were stimulated to divide by the addition of a mitogen (e.g., phytohemagglutinin, PHA). Mitotic activity began at about 40 hours after PHA stimulation and reached a maximum at approximately 3 days.

Xylanase X3 was mixed with cultures of human peripheral lymphocytes both in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver; S-9 mix). This assay consisted of a preliminary toxicity (dose range finding) assay and two main assays. Ten concentrations of Xylanase X3 were used in the preliminary assay and at least 4 dose levels were then selected for the definitive assay with the highest dose level clearly inducing a toxic effect (50% reduction in mitotic index). Cytotoxicity is characterized by the percentage of mitotic suppression in comparison to the controls. In the absence of cytotoxicity, the highest dose selected would be 5000 µg TP/ml, as recommended by the OECD guideline. All dose levels were expressed in terms of total protein.

In the preliminary assay, all cultures with or without S-9 mix were treated for 4 hours and continuously for 20 hours in the absence of S-9 mix. In the definitive assay, cultures with and without S-9 mix were exposed to the test article for 4 hours, and continuously for 20 hours in the absence of S-9 mix. For the preliminary and the definitive assays, cells were collected 20 hours (1.5 normal cell cycles) after initiation of treatment. Two hours prior to harvest, Colcemid was added to the cultures at a final concentration of 0.1 µg/ml to arrest mitosis.

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

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

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

b. Results

In the preliminary assay, the dose levels ranged from 0.5 to 5000 µg TP/ml. Exposure period was 4 hours for both cultures with and without S9 mix, and continuously for 20 hours in the absence of S-9 mix. All cells were harvested after 20 hours after treatment initiation. No visible precipitation of the test material in the culture medium was observed. Substantial toxicity (at least 50% reduction in mitotic index relative to the vehicle control) was not observed at any dose level including the 5000 µg/ml dose level in both the non-activated and activated groups. Based on those findings, dose levels ranging from 500 to 5000 µg/ml were used in the definitive assays.

In the definitive assay, substantial toxicity (at least 50% reduction in mitotic index relative to the vehicle control) was not observed in both non-activated and activated groups at any dose level including the highest dose tested, 5000 µg TP/ml. At the highest test dose evaluated microscopically for chromosome aberrations, 5000 µg/ml, mitotic index was 14% reduced relative to the vehicle control. Based on these findings, the doses chosen for microscopic analysis were 1000, 2500 and 5000 µg TP/ml.

The test article did not induce any statistically significant increases in the frequency of cells with aberrations in either the presence or absence of S-9 mix. No increase in polyploidy metaphases was noticed. Significant increases in aberrant metaphases were demonstrated with the positive controls demonstrating the sensitivity of the tests and the efficacy of the S-9 mix.

c. Evaluation

Under the conditions of this test, Xylanase X3 did not induce chromosomal aberrations (both structural and numerical) in this *in vitro* cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation up to the highest concentration 5000 µg TP/ml recommended by guidelines. All of the vehicle control cultures had frequencies of cells with chromosomal aberrations within the expected range. The positive control items inducted statistically significant increases in the frequency of cells with aberrations.

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

a. Procedure

The objective of this study was to investigate the potential of X3 Xylanase to induce systemic toxicity after repeated daily oral administration to Charles River CD rats of both sexes for 90 continuous days. Groups of 10 animals per sex were treated by oral gavage with 0 (distilled water), 100, 300 or 1000 mg TOS/kg bw/day. The dosing volume was 10 ml/kg bw/day.

Animals of the same sex were housed in groups of two to three in solid floor polypropylene cages with stainless steel mesh lids and softwood bedding (non-aromatic) with access to water via an automatic system and feed *ad libitum*. For environmental enrichment, the animals were provided a supply of wooden chew blocks and cardboard fun tunnels. All groups were housed under controlled temperature, humidity and lighting conditions.

All animals were observed daily for mortality and signs of morbidity. Body weight and feed consumption were recorded weekly. Water consumption was recorded twice weekly for each cage. Ophthalmologic examination was performed on all animals prior to study initiation and in

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

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

b. Results
No treatment-related deaths were noted during the 13-week period. There were no treatment-related changes in body weights, feed consumption and water intake. Hematology and clinical chemistry conducted after 13 weeks of treatment did not reveal any adverse effects.

There were no biological or statistical differences between the control and treated groups with respect to clinical observation, feed consumption, water consumption, ophthalmologic examinations, body weights, and body weight gains. There were no treatment-related changes in hematology and clinical chemistry at the end of week 13. There were no differences in the functional observation battery, grip strength and locomotor activity assays between treated and control animals.

At necropsy, there were no treatment related findings on organ weights, macroscopic findings and histopathologic examinations. All microscopic findings were considered to be within the background incidence of findings reported in this age and strain of laboratory animals.

c. Evaluation and conclusion
Daily administration of X3-Xylanase by oral gavage to CD rats at doses of 0, 100, 300 or 1000 mg TOS/kg bw/day for 90 consecutive days did not result in treatment-related effects on clinical observations, feed consumption, body weight changes, hematology, clinical chemistry, urinalysis, organ weights, functional observation, grip strength or locomotor activities. No macroscopic or microscopic changes could be attributed to treatment. Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established at the highest dose tested, 1000 mg TOS/kg bw/day.

In conclusion, the safety of Xylanase X3 was assessed in a battery of toxicology studies investigating its genotoxic and systemic toxicity potential. Under the conditions of the mutagenicity assays Xylanase is not a mutagen or clastogen. Daily administration of Xylanase X3 by gavage for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at 1000 mg TOS/kg bw/day.

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 1000 mg TOS/kg bw/day. The study was designed based on OECD guideline No. 408 and conducted in compliance with both the FDA Good Laboratory Practice Regulations and the OECD Good Laboratory Practice. Since human exposure to Xylanase X3 is through oral ingestion, selection of this NOAEL is thus appropriate.

NOAEL = 1000 mg TOS/kg bw/day

7.4.2 Human Exposure to Xylanase X3

To estimate worst-case exposure to Xylanase X3, the relatively conservative Budget method was used. According to the Budget method (Douglass et al., 1997) the solid food consumption is assumed to be 100 kcal/kg and 50 kcal/kg for children and adults, respectively. Using 60 kg as a default body weight for adults, the maximum lifetime energy intake of 50 kcal/kg bw/day for solid foods corresponds to 25 g/kg bw/day.

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

Estimated intake of the Xylanase X3

Xylanase X3 will be used as a processing aid where the enzyme is not present in the final food or present as inactive residue in negligible amounts.

As per section 1.4 'Enzyme activity' the specific activity of the subject xylanase enzyme is 1974 XBU/mg active protein and per section 4.3 'Total Organic Solid (TOS)', TOS for Xylanase X3 is 42.13 % for an activity of 102,433 XBU/g. This creates a TOS/active enzyme conversion ratio of 8.12 mg TOS/mg active protein.

Estimation of daily consumption of Xylanase X3 from its potential uses in major commodities is based on the Budget method as proposed by EFSA (2011):

Brewing:

Xylanase X3 is used in the brewing industry at 22.5 g active protein/MT grist (= 22.5 mg active protein/kg grist). It is assumed the 17 kg grist gives 100 L beer and that 100% Xylanase X3 remains in the beer after processing.

The maximum concentration of Xylanase X3 found in beer:

Max application level grist	22.5	mg active protein /kg grist
Max application level grist	185	mg TOS/kg grist
Yield of beer from grist	5.88	L/kg grist
Enzyme in beer	31.5	mg TOS/L beer
Enzyme in beer	31.5	µg TOS/mL beer

Potable Alcohol:

Since the application rate of Xylanase X3 in brewing is higher than that of potable alcohol and the resulting exposure is much higher (due to lack of the distillation process), the higher exposure from brewing will be used in this risk assessment to represent a worst case scenario.

Cereal beverage:

Xylanase X3 is used in the cereal beverage industry at 3.75 g active protein/MT grist (= 3.75 mg active protein/kg grist). It is assumed the 25 kg grist gives 100 L beverage and that 100% Xylanase X3 remains in the bevereage after processing.

The maximum concentration of Xylanase X3 found in cereal beverage:

max application level grist	3.75	mg active enzyme protein /kg grist
max application level grist	30.4	mg TOS/kg grist
yield of beer from grist	4.0	L/kg grist
enzyme in beer	7.6	mg TOS/L cereal beverage
enzyme in beer	7.6	µg TOS/mL cereal beverage

Bakery

Xylanase X3 is used in baking industry (including yeast raised bread and tortilla) at a maximum of 22.5 mg active enzyme protein/kg flour. It is assumed that 1kg flour is used to produce 1.4 kg of bread and that 100% Xylanase X3 remains in the bread after processing.

The maximum concentration of Xylanase X3 found in bread:

Max application level baking	22.5	mg active enzyme protein/kg flour
Max application level baking	185	mg TOS/kg flour
1/Yield of bread from flour	0.71	kg flour/kg baking good
Enzyme in baking goods	131.4	mg TOS/kg baking good
Enzyme in baking goods	131.4	µg TOS/g baking good

According to the Budget method (Douglass et al., 1997) the solid food consumption is assumed to be 100 kcal/kg and 50 kcal/kg for children and adults, respectively. Using 60 kg as a default body weight for adults, the maximum lifetime energy intake of 50 kcal/kg bw/day for solid foods corresponds to 25 g/kg bw/day.

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

- ***Liquid food contribution***

Since exposure of Xylanase X3 from cereal beverages is lower than that from brewing, the brewing application will be used to represent a worst case scenario. As presented above enzyme exposure from distilling is complete disregarded as result of the distillation process.

Beverage (non milk) intake	100	ml/kg bw/day
Enzyme Processed beverage intake (25%)	25	ml/kg bw/day

- ***Solid food contribution***

Bakery application is the sole solid food application represented and the max dosage used in this application is used for representation of worst case scenario.

Solid food intake	25	g/kg bw/day
Processed food treated with enzyme (50%)	12.5	g/kg bw/day

The maximum daily intake of Xylanase X3 from liquid (Brewing, Potable alcohol, Cereal Beverages) and solid foods (Bakery goods) is:

TMDI $_{\text{Liquid food}}$	787	µg TOS/kg bw/day
TMDI $_{\text{Solid food}}$	1642	µg TOS/kg bw/day
TMDI total	2428	µg TOS/kg bw/day

Determination of the margin of safety

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

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

$$\text{Margin of Safety} = \frac{1000 \text{ mg TOS/kg bw/day}}{2.428 \text{ mg TOS/kg bw/day}}$$

Margin of Safety = 412

7.4.3 Conclusion

The safety of Xylanase X3 is assessed in a battery of toxicology studies investigating its irritation, acute ingestion, genotoxic and systemic toxicity potential. Xylanase X3 is not an eye and skin irritant (Note: these studies were done as part of assessing worker safety). A battery of genotoxicity assays was conducted and under the conditions of these assays Xylanase X3 is not a mutagen, a clastogen, or an aneugen.

Daily administration of Xylanase X3 by gavage for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at 1000 mg TOS/kg bw/day.

Even under the worst case scenario that Xylanase X3 is applied at the maximum rate and the enzyme is not destroyed and/or removed during processing, the use of Xylanase X3 in brewing, cereal beverages, manufacture of potable alcohol and bakery application is not expected to result in adverse effects to humans. Under the conditions of these safety/toxicology studies, a margin of safety of 412 exists between the systemic NOAEL of 1000 mg TOS/kg bw/ and the estimated maximum daily human cumulative exposure level of 2.428 mg TOS/kg bw/day.

8. BASIS FOR GENERAL RECOGNITION OF SAFETY

As noted in the Safety sections above, *Trichoderma reesei*, and enzyme preparations derived therefrom, including cellulase, pectin lyase, glucoamylase, beta-glucanase, xylanase, chymosin, transglucosidase, and acid fungal protease enzyme preparations, are well recognized by qualified experts as being safe. Published literature, government laws and regulations, reviews by expert panels such as FAO/WHO JECFA, as well as DuPont Industrial Biosciences' own unpublished safety studies, support such a conclusion.

T. reesei 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 Xylanase X3 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 Xylanase X3 produced by the production strain described in this submission established a NOAEL at 1000 mg TOS/kg bw. Based on a worst-case scenario, the use of Xylanase X3 in brewing and manufacture of potable alcohol and cereal beverage, and bakery application is not expected to result in adverse effects to humans. A safety margin of 412 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 Xylanase X3 from *Trichoderma reesei* strain Brew2#21 is safe and suitable for use in grain processing brewing, potable alcohol and cereal beverage manufacture, and bakery application. 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 8) for its intended uses.

9. 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.

Bissett, J. 1984. A revision of the genus *Trichoderma*. I. Section *Longibrachiatum* sect. Nov. Can. J. Bot. 62: 924-931.

Blumenthal. C. 2004. Production of toxic metabolites in *Aspergillus niger, Aspergillus oryzae*, and *Trichoderma reesei*: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. Regul. Toxicol. Pharmacol. 39: 214-228.

Brückner, H. and Graf, H. 1983. Paracelsin, a peptide antibiotic containing alpha-aminoisobutyric acid, isolated from *Trichoderma reesei* Simmons. Part A. Experientia 39:528-530.

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.

Dugan, F.M. 1998. A Note on *Trichoderma reesei* and *T. longibrachiatum*, United States Federation for Culture Collections Newsletter. 28(2): 1- 2.

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.

GRAS Notice 230. 2008. Chymosin enzyme preparation from *Trichoderma reesei* expressing the bovine prochymosin B gene. http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=230.

GRAS Notice 315. 2010. Transglucosidase enzyme preparation from *Trichoderma reesei* expressing the gene encoding the transglucosidase from *Aspergillus niger*. http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=315

GRAS Notice 333. 2010. Acid fungal protease enzyme preparation from *Trichoderma reesei* expressing the gene encoding acid fungal protease from *T. reesei*. http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=333

GRAS Notice 372. 2011. Glucoamylase enzyme preparation from *Trichoderma reesei* expressing the glucoamylase gene from *T. reesei* (glucoamylase enzyme preparation) http://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&sort=GRN_No&order=DESC&startrow=1&type=basic&search=372

GRAS Notice 89. 2001. Five enzyme preparations from *Aspergillus niger*: Carbohydrase enzyme preparation, catalase enzyme preparation, glucose oxidase enzyme preparation, pectinase enzyme preparation, and protease enzyme preparation http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=89

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.

Kuhls, K, Lieckfeldt, E., Samuels, G.J., Kovacs, W., Meyer, W., Petrini, O., Gams, W., Börner, T. and Kubicek, C.P. 1996. Molecular evidence that the asexual industrial fungus *Trichoderma*

reesei is a clonal derivative of the ascomycete *Hypocrea jecorina*, Proc. Natl. Acad. Sci., USA 93: 7755-7760.

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

Meyer, W., Morawetz, R., Borner, T. and Kubicek, C. P. 1992. The use of DNA-fingerprint analysis in the classification of some species of the *Trichoderma* aggregate. Curr. Genet. 21: 27-30.

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

Nevalainen, H., Suominen, P., and Taimisto, K. 1994. On the safety of *Trichoderma reesei*, J. Biotechnol. 37: 193-200.

Olempska-Beer, Z.S., Merker, R.I., Ditto, M.D., and DiNovi, M.J. ,2006. Food-processing enzymes from recombinant microorganisms—a review. Regul. Toxicol. Pharmacol. 45: 144-158.

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.

Schuster, E.; Dunn-Coleman, N.; Frisvad, J.C.; van Dijck, P.W.M. (2002). On the safety of Aspergillus niger — a review. Appl. Microbiol. Biotechnol. 59, 426–435.

Sheir-Neiss, G. and Montenecourt, B.S. 1984. Characterization of the secreted cellulases of *Trichoderma reesei* wild type and mutants during controlled fermentations. Appl. Microbiol. Biotechnol. 20: 46-53.

Solfrizzo, M., Altomare, C., Visconti, A., Bottalico, A. and Perrone, G. 1994. Detection of peptaibols and their hydrolysis products in cultures of *Trichoderma* species. Nat. Toxins 2: 360-365.

US EPA Biotechnology Program Under Toxic Substances Control Act (TSCA) *Aspergillus niger* Final Risk Assessment (February 1997), available at: http://www.epa.gov/biotech_rule/pubs/pdf/fra006.pdf

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

10. LIST OF APPENDICES

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Appendix 1 – 21 CFR 170.30

[Code of Federal Regulations]

[Title 21, Volume 3]

[Revised as of April 1, 2005]

From the U.S. Government Printing Office via GPO Access

[CITE: 21CFR170.30]

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TITLE 21--FOOD AND DRUGS

CHAPTER I--FOOD AND DRUG ADMINISTRATION, DEPARTMENT OF HEALTH AND HUMAN

SERVICES (CONTINUED)

PART 170_FOOD ADDITIVES--Table of Contents

Subpart B_Food Additive Safety

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

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

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

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

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

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

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

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

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

(2) Any substance of natural biological origin that has been widely consumed for its nutrient properties in the United States prior to January 1, 1958, without known detrimental effect, for which no health hazard is known, that has had significant alteration of composition by breeding or selection after January 1, 1958, where the change may be reasonably expected to alter the nutritive value or the concentration of toxic constituents.

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

(4) Reaction products of GRAS substances.

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

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

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

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

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

741-6030, or go to: http://www.archives.gov/federal--register/code--of--federal--regulations/ibr--locations.html.

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

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

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

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

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

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

[42 FR 14483, Mar. 15, 1977, as amended at 49 FR 5610, Feb. 14, 1984; 53

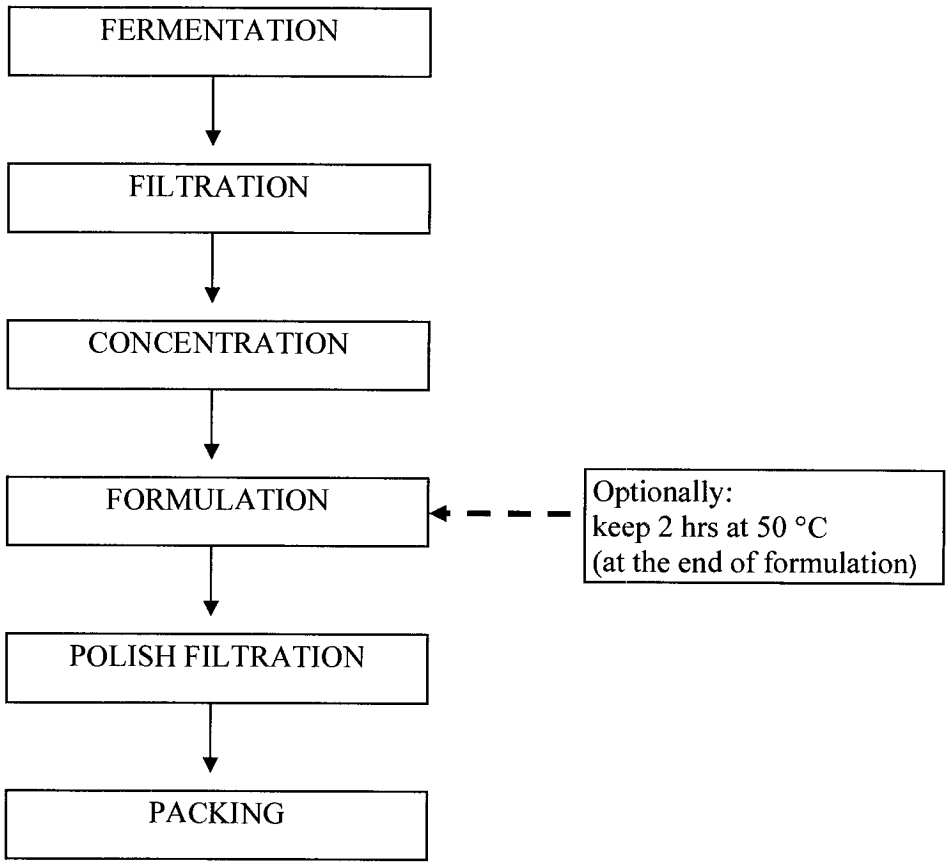
FR 16546, May 10, 1988]

Appendix 2 – The amino acid sequence of Xylanase X3

Predicted signal sequence in bold.

MVQIKAAALAMLFASHVLSEPIEPRQASVSIDTKFKAHGKKYLGNIGDQYTLTKNSKT
PAIIKADFGALTPENSMKWDATEPSRGQFSFSGSDYLVNFAQSNNKLIRGHTLVWHSQL
PSWVQAITDKNTLIEVMKNHITTVMQHYKGKIYAWDVVNEIFNEDGSLRDSVFYQVIGE
DYVRIAFETARAADPNAKLYINDYNLDSASYPKLTGMVSHVKKWIEAGIPIDGIGSQTHL
SAGGGAGISGALNALAGAGTKEIAVTELDIAGASSTDYVEVVEACLDQPKCIGITVWGV
ADPDSWRSSSTPLLFDSNYNPKPAYTAIANAL

Appendix 3- The Manufacturing Process



Appendix 4 – CERTIFICATE OF ANALYSIS - Xylanase X3 Intermediate

Property	Lot number 20121024	Lot number 20121025	Lot number 20128109
ENZYME ACTIVITIES			
Xylanase	130932 XBU/g	96434 XBU/g	95058 XBU/g
MICROBIAL ANALYSIS			
Total Viable Count	3 CFU/ml	1 CFU/ml	<1 CFU/ml
Total Coliforms	<1 CFU/ml	<1 CFU/ml	<1 CFU/ml
E. coli	NEG/25 ml	NEG/25 ml	NEG/25 ml
Salmonella	NEG/25 ml	NEG/25 ml	NEG/25 ml
Lactic Acid Bacteria	<1 CFU/ml	<1 CFU/ml	<1 CFU/ml
Production strain	NEG/ml	NEG/ml	NEG/ml
Antimicrobial Activity	NEG/ml	NEG/ml	NEG/ml
PHYSICAL PROPERTIES			
pH	4.9	4.9	4.9
Appearance	Pass	Pass	Pass
OTHER ASSAYS			
Heavy Metals, as Pb	<30 mg/kg	<30 mg/kg	<30 mg/kg
Arsenic	<3 mg/kg	<3 mg/kg	<3 mg/kg
Cadmium	<0.5 mg/kg	<0.5 mg/kg	<0.5 mg/kg
Mercury	<0.5 mg/kg	<0.5 mg/kg	<0.5 mg/kg
Lead	<5 mg/kg	<5 mg/kg	<5 mg/kg
Mycotoxins, T-2	NEG	NEG	NEG

Appendix 5: Pariza & Johnson Decision Tree on *A. niger* Xylanase X3 expressed in *T. reesei*

1. Is the production strain[1] genetically modified[2,3]?
Yes → go to 2.

2. Is the production strain modified using rDNA techniques?
Yes → go to 3a.

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

Yes, xylanase has been used for years in food processing and animal feed. The *Aspergillus niger* xylanase X3 is new as an isolate expressed in *Trichoderma reesei* for use in food processing, but has been present at lower amounts in other enzyme preparations from its donor organism → go to 3c.

3c. Is the test article free of transferable antibiotic resistance gene DNA[7]?

Yes, no antibiotic resistance genes were used in the construction → go to 3e.

3e. Is all other introduced DNA well characterised and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products?

Yes, inserted DNA is well characterized and free of unsafe attributes. Go to 4.

4. Is the introduced DNA randomly integrated into the chromosome? Yes. *Go to 5.*

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

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

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

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

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

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

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

5. **Is the production strain sufficiently well characterised 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?** Yes. The inserted DNA is well characterized. The production strain does not produce toxic metabolites of concern as confirmed by T-2 toxin analysis. *Go to 6.*

6. **Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure[8]?** Yes. The *T. reesei* RL-P37 safe lineage is established as presented in Appendix 6. Its safety as a production host and methods of modification are well documented, and their safety have been confirmed through repeated toxicology testing (see Appendix 6).

Conclusion: Article is accepted.

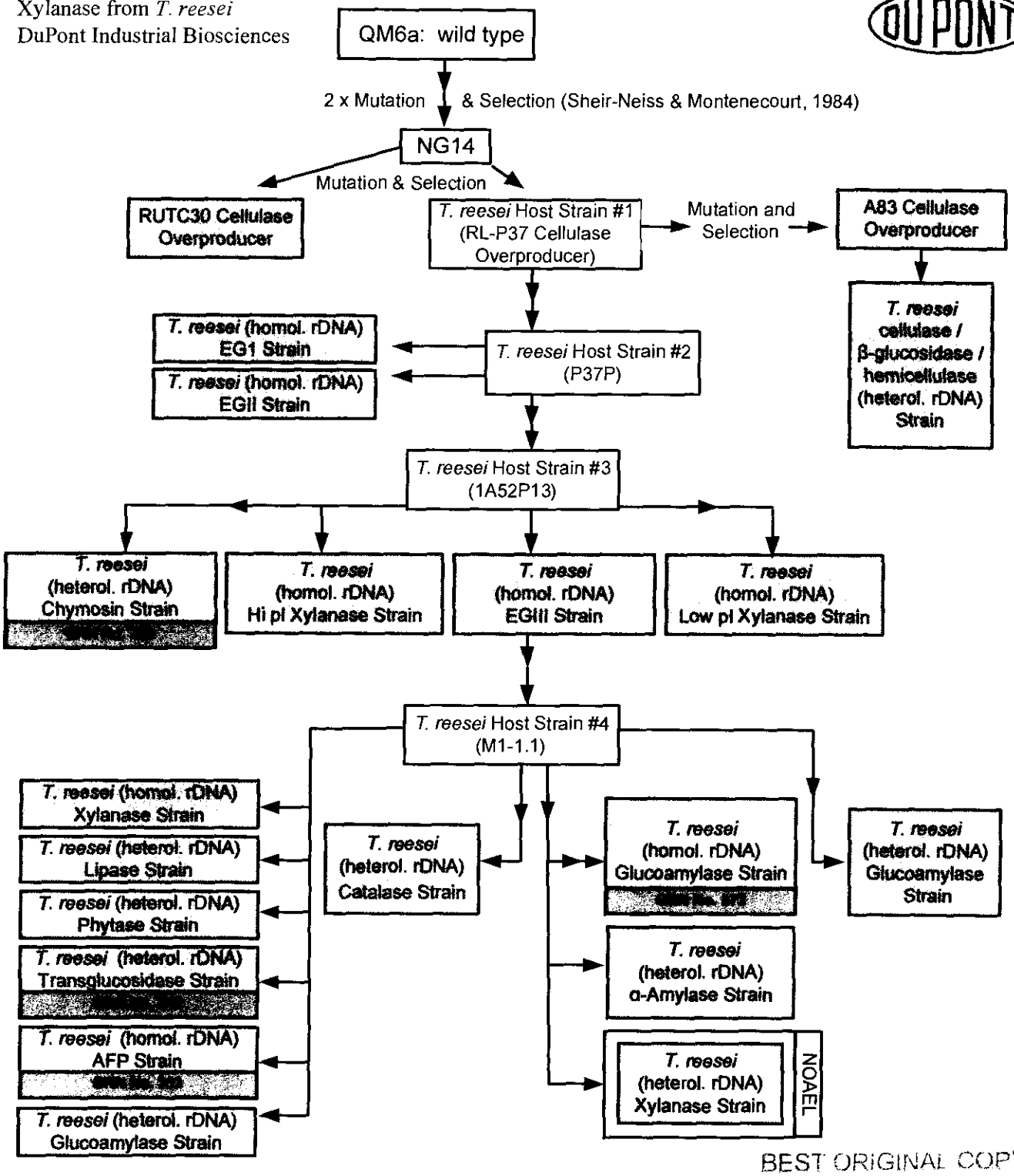
Based on the publicly available scientific data from the literature and additional supporting data generated by DuPont, the company has concluded that *Aspergillus niger* Xylanase X3 expressed in *Trichoderma reesei* strain Brew2#21 is safe and suitable for use in brewing application, potable alcohol manufacture, cereal beverage production and bakery and is Generally Recognized as Safe (GRAS) for those uses.

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

Appendix 6 – *T. reesei* Strain Lineage and Summary of Safety Studies

T. reesei Strain Lineage

Note: The number of steps between strains is generally more than is shown. Text boxes in ORANGE color indicate toxicology testing was conducted on the strain and/or product of it as described below; text box in border indicates the strain is the subject of this submission.



BEST ORIGINAL COPY

Most enzymes derived from this Safe Strain Lineage were determined to be GRAS for their intended use, with GRAS Notices reviewed by the US FDA for enzymes from strains designated with green horizontal banners indicating the GRAS Notice number.

The subject strain of this submission is the Xylanase producing strain indicated by the yellow border.

The safety of the xylanase enzyme is fully supported by repeated testing of other enzymes produced by members of this Safe Strain Lineage. The orange-colored boxes indicate strains for which we conducted toxicology tests.

The NOAEL for this xylanase is used to calculate its safety margin in the intended uses.

Toxicology Test Summaries

The safety of the 18 enzyme preparations derived from the 18 recombinant production strains were assessed in a number of toxicology tests as shown in the table below. The table also includes the toxicology tests for two non-recombinant *T. reesei* strains (RUT C30 and A83) and/or product derived from them. All enzyme preparations were found to be non-toxic, non-mutagenic and not clastogenic.

PRODUCTION ORGANISM	ENZYME	TOXICOLOGY TEST	RESULT
I. *T. reesei* A83 (traditionally modified)	cellulase	Pathogenicity study, rats	Non-pathogenic Non-toxicogenic
		91-day subchronic oral toxicity study, rats	No adverse effect
		Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosomal aberration assay, human lymphocytes	Not clastogenic
II. *T. reesei* RUT C30 (traditionally modified)	cellulase	91-day subchronic oral toxicity study, rats	No adverse effects
		Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosomal aberration assay, human lymphocytes	Not clastogenic
III. T. reesei (homologous rDNA)	Endoglucanase I	14-day subacute study, rats	No adverse effects
		Pathogenicity study, rats	Non pathogenic
		91-day subchronic oral toxicity study, rats	No adverse effects
		In vitro chromosome assay, human lymphocytes	Not clastogenic
IV. *T. reesei* (homologous rDNA)	High pI Xylanase	91-day subchronic oral toxicity study, rats	No adverse effects
		Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosome assay, Chinese hamster ovary cells	Not clastogenic
V. T. reesei (homologous rDNA)	Endoglucanase II	90-day subchronic oral toxicity study, rats	No adverse effects

		In vitro chromosomal aberration assay, human lymphocytes	Not clastogenic
		Bacterial reverse mutation assay	Not mutagenic
VI. T. reesei (homologous rDNA)	Endoglucanase III	28-day subacute oral toxicity study, rats	No adverse effects
		Bacterial reverse mutation assay	Not mutagenic
VII. T. reesei (homologous rDNA)	Low pI Xylanase	91-day subchronic oral toxicity study, rats	No adverse effects
		Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosomal aberration assay, human lymphocytes	Not clastogenic
VIII. T. reesei (homologous rDNA)	Xylanase	91-day subchronic oral toxicity study, rats	No adverse effects
		Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosomal aberration assay, human lymphocytes	Not clastogenic
IX. *T. reesei* (homologous rDNA)	Protease	91-day subchronic oral toxicity study, rats	No adverse effects
		Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosomal aberration assay, human lymphocytes	Not clastogenic
X. *T. reesei* (heterologous rDNA)	Phosphatase (4-Phytase)	91-day subchronic oral toxicity study, rats	No adverse effects
		Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosome assay, human lymphocytes	Not clastogenic
XI. *T. reesei* (heterologous rDNA)	Chymosin	Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosomal aberration assay, human lymphocytes	Not clastogenic
		91-day subchronic study, rats	No adverse effects detected

XII. *T. reesei* (heterologous rDNA)	Trans-glucosidase	Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosomal aberration assay, human lymphocytes	Not clastogenic
		18-week subchronic study in rats	No adverse effects
XIII. *T. reesei* (homologous rDNA)	Glucoamylase	Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosome assay, human lymphocytes	Not clastogenic
		91-day subchronic oral toxicity study, rats	No adverse effects
XIV. *T. reesei* (heterologous rDNA)	Lipase	Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosomal aberration assay, human lymphocytes	Not clastogenic
		91-day subchronic oral toxicity study, rats	No adverse effects
XV. *T. reesei* (heterologous rDNA)	alpha-amylase	Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosomal aberration assay, human lymphocytes	Not clastogenic
		91-day subchronic oral toxicity study, rats	No adverse effects
XVI. *T. reesei* (heterologous rDNA)	Cellulase, beta-glucosidase, hemicellulases	Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosomal aberration assay, human lymphocytes	Not clastogenic
		91-day subchronic oral toxicity study, rats	No adverse effects
XVII. *T. reesei* (heterologous rDNA)	glucoamylase	Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosomal aberration assay, human lymphocytes	Not clastogenic
		91-day subchronic oral toxicity study, rats	No adverse effects
XVIII. *T. reesei* (heterologous rDNA)	Xylanase	Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosomal aberration assay, human lymphocytes	Not clastogenic
		91-day subchronic oral toxicity study, rats	No adverse effects

T. reesei (heterologous rDNA)	Catalase	Bacterial Reverse Mutation Assay	Not mutagenic
		In vitro chromosomal aberration assay, human lymphocytes	Not clastogenic
		91-day subchronic oral toxicity study, rats	No adverse effects
T. reesei (heterologous rDNA)	Glucoamylase	Bacterial Reverse Mutation Assay	Not mutagenic
		In vitro chromosomal aberration assay, human lymphocytes	Not clastogenic
		91-day subchronic oral toxicity study, rats	No adverse effects

I. Cellulase from *T. reesei* A83 (traditionally modified)

A. Pathogenicity study in rats

This study was conducted in compliance with the Good Laboratory Practice Standards as published by the OECD Guidelines, the US FDA and the US EPA. In this investigation, *Trichoderma reesei* strain A83 was administered as a single intraperitoneal dose of 2.2 x 10^7 cfu to 15 male and 15 female Sprague Dawley rats. Five additional animals per sex were used as the untreated control group. Groups of animals were killed on Days 4, 8, 15, 22 and 32 for microbial enumeration and necropsy observations (BioReliance, 1998). No deaths occurred and no adverse clinical observations were noted. No treatment related differences in body weights or body weight changes were observed at any time point, when compared to controls. No abnormal findings were noted at necropsy. By Day 15, *T. reesei* was detected in only the spleen and liver of both males and females and, by Day 22, in only the spleen of both male and female rats. Total clearance of the test microbe from the spleen was achieved by Day 32. Under the conditions of this investigation, *T. reesei* strain A83 was not found to be toxic or pathogenic in rats.

B. 91-day subchronic feeding study in rats

This study was conducted in accordance with OECD Guideline 408 in CD rats. Groups of 20 male and female CD rats were fed with 4 different concentrations of the test material in the diet for 13 consecutive weeks (IRDC, 1990).

No mortalities were recorded throughout the entire investigation. There were no treatment-related adverse effects on any of the parameters (systemic toxicity, food consumption, opthalmology, clinical chemistry, body weight, organ weight, urine analysis, hematology, necropsy, and histopathology) monitored in this study for rats treated with *T. reesei* cellulase. Based upon these findings, it was concluded that the treatment of male and female rats with cellulase from *T. reesei* did not result in toxicity at the highest dosage in the diet.

A NOEL (No Observed Effect Level) was established at the highest dosage tested.

C. Bacterial reverse mutation assay (Ames assay)

The test material, a cellulase from *T. reesei* A83 was tested in four strains of *Salmonella typhimurium* (TA98, TA100, TA 1535 and TA1537) and *Escherichia coli* tester strain WP2 *uvr*A in the presence and absence of metabolic activation (BioReliance, 2002). The assay was conducted in accordance with OECD Guideline 471.

In the first assay, eight dose levels ranging from 2.5 to 5,000 µg/plate were used and no positive mutagenic response was observed at any dose level in any of the exposure groups. In the confirmatory assay, dose levels ranging from 75 to 5,000 µg/plate were used. No positive mutagenic response was observed in both the presence and absence of

metabolic activation. Under the conditions of this assay, *T. reesei* cellulase was not a mutagen.

D. *In vitro* chromosomal aberration assay with human peripheral blood lymphocytes

The test material, a cellulase from *T. reesei* A83 was tested in the *in vitro* mammalian chromosome aberration test using human peripheral blood lymphocytes (HPBL) in both the absence and presence of metabolic activation (BioReliance, 2002). The assay was conducted in accordance with OECD Guideline 473.

In a preliminary toxicity assay, HPBL cells were exposed to 9 concentrations of the test material ranging from 0.5 to 5000 µg/ml and solvent controls in both the presence and absence of metabolic activation. Cytotoxicity was not observed at any dose level in any of the exposure groups. Based on these findings, dose levels of 625, 1250, 2500 and 5000 µg/ml were selected for the chromosome aberration assay. In the main chromosome aberration assay, HPBL cells were incubated with the test material for 4 and 20 hours in the non-activated test system and for 4 hours in the activated test system. All cells were harvested 20 hours after treatment. Mitomycin C and cyclophosphamide served as positive controls. No statistically or biologically significant increases in the number of cells with aberrations (structural or numerical) were noted for the test material treated cells. Under the conditions of this investigation, cellulase from *T. reesei* did not induce any clastogenic or aneugenic effects in cultured human lymphocytes, either with or without metabolic activation.

II. Cellulase from *T. reesei* RUT C30 (traditionally modified)

A. 90-day feeding study, rats

A 13-week dietary feeding study was conducted in rats with the unformulated cellulase concentrate. Three groups of male and female CD rats received cellulase preparation in the diet at 3 concentrations for 90 consecutive days. Body weights and body weight changes were recorded weekly during the study. Hematology, clinical chemistry, urine analysis, necropsy and pathological observation were recorded at study termination.

The only adverse effect noted in this study was a slight increase in urinal acidity and specific gravity noted in males. No evidence of toxicity was noted in this study relative to body weight, body weight changes, organ weights, clinical observation, food consumption, hematology, clinical chemistry, ophthalmology, necropsy and pathological observations up to and including the highest dose tested.

Under the conditions of this experiment, cellulase was non-toxic to rats and the NOEL (No observed effect level) was established at the highest dose.

B. Bacterial reverse mutation assay

In a bacterial reverse mutation assay (Ames assay), cellulase was tested at 8, 40, 200, 1000 or 5000 ug/plate for its ability to induce mutation in five *Salmonella* strains and one *Escherischia coli* strain. No positive mutagenic response was observed in both the presence and absence of metabolic activation up to and including the highest dose tested, 5000 ug/plate. Under the conditions of this investigation, cellulase from *Trichoderma reesei* RUT C30 was not mutagenic in the bacterial reverse mutation assay using *Salmonella* and *E. Coli* Cellulase in both the presence and absence of metabolic activation.

C. *In vitro* chromosomal aberration assay with human lymphocytes

The clastogenic activity of cellulase from *T.reesei* RUT C30 was investigated in a human peripheral blood lymphocyte (HPBL) assay. Cellulase was tested at 0, 500, 1,667 or 5,000 ul/ml in the absence of metabolic activation and at 0, 1,000, 3,333, or 10,000 ug/ml in the presence of metabolic activation. Whole blood cultures from two donors were treated with cellulase for 3 hours in the presence of S9 and continuously for 24 hours in the absence of S9.

No statistical significant increases in chromosome aberrations were noted. Under the conditions of this assay, cellulase was not clastogenic to HPBL in both the presence and absence of metabolic activation.

III. Endoglucanase I from *T. reesei* (homologous rDNA)

A. Pathogenicity study in rats

This study was conducted in accordance with the US. EPA Microbial Pesticide Test Guideline OPPTS 885.3200 and the US. EPA Good Laboratory Practice Standards (40CFR.160). In this investigation, the pathogenicity potential of *T. reesei* Endoglucanase I production strain was tested in male and female CD rats following an acute intraperitoneal injection of 5.6×10^6 colony forming units (cfu). Groups of animals of both sexes were sacrificed on Days 0, 7, 21, and 35 after injection of the test substance for microbial enumeration. The results were compared to those obtained from heat-killed test substance group, naïve control group and shelf control group (IITRI, 2000). No deaths occurred and no adverse clinical observations were noted. The major necropsy findings were enlarged spleens noted in treated females on Days 7 and 21 and in treated males on Day 21. On Day 7, a significant decrease in relative liver weight was noted in treated males but was no longer observed on subsequent necropsy days. A statistical difference in body weight was noted in treated males between Days 0-7 but all animals showed complete recovery thereafter. By Day 21, *T. reesei* was detected in the peritoneal lavage fluid and masses of treated male rats and from the liver and peritoneal masses of treated female rats. By Day 35, the test substance was detected only from the peritoneal masses. Complete clearance was estimated to be 67 and 43 days for male and female

rats, respectively. Under the conditions of this investigation, *T. reesei* Endoglucanase I production strainwas not found to be toxic or pathogenic in rats.

B. 14-day oral feeding study in rats

This study was conducted in accordance with OECD Guideline 408 in rats. Endoglucanase I was administered orally by gavage to groups of male and female Charles River CrlCD® BR rats at a dosage volume of 10 ml/kg body weight for 14 days at 4 different concentrations for 14 consecutive days (IRDC, 1993). There were no adverse effects on any parameter monitored in this study for rats treated with the enzyme. It was concluded that the treatment of male and female rats with the enzyme preparation did not produce evidence of systemic toxicity in any of the parameters that were monitored.

C. 91-day subchronic oral study in rats

A 13-week oral gavage study with endoglucanase from *T. reesei* was conducted in accordance with OECD Guideline 408 in CD rats. Groups of ten male and females rats each were exposed to 3 different concentrations of the test material by gavage in a constant volume of 5 ml/kg/day. Control animals received deionized water following the same treatment regimen (MDS Pharma, 2002).

No mortalities were recorded throughout the entire investigation. There were no treatment-related adverse effects on any of the parameters (systemic toxicity, food consumption, opthalmology, clinical chemistry, body weight, organ weight, urine analysis, hematology, necropsy, and histopathology) monitored in this study for rats treated with endoglucanase from *T. reesei*. There were no differences in behavioral test (open field) and functional tests (gripping reflex, startle reflex) conducted at study termination between the control and treated groups. Based upon these findings, it was concluded that the treatment of male and female rats with the endoglucanase from *T. reesei* did not result in toxicity up to and including the highest dosage tested. A NOEL (No Observed Effect Level) was established at the highest dosage tested.

D. *In vitro* chromosomal aberration assay with human peripheral blood lymphocytes

Endoglucanase I from *T. reesei* was tested in the *in vitro* mammalian chromosome aberration test using human peripheral blood lymphocytes (HPBL) in both the absence and presence of metabolic activation. The assay was conducted in accordance with OECD Guideline 473.

In a preliminary toxicity assay, HPBL cells were exposed to 9 concentrations of the test material ranging from 0.5 to 5000 µg/ml as well as solvent controls in both the presence and absence of metabolic activation. Cytotoxicity was not observed at any dose level in any of the exposure groups. Based on these findings, dose levels of 625, 1250, 2500 and 5000 µg/ml were selected for the chromosome aberration assay.

In the main chromosome aberration assay, HPBL cells were incubated with the test material for 4 and 20 hours in the non-activated test system and for 4 hours in the activated test system. All cells were harvested 20 hours after treatment. Mitomycin C and cyclophosphamide served as positive controls. In the non-activated 4 hour exposure group, mitotic inhibition was 10% relative to the solvent control at the 5,000 µg/ml dose level. A mitotic inhibition of 7% was also noted at the 5,000 µg/ml dose level in the activated assay. However, the percentage of HPBL cells with structural and numerical aberrations in both 5,000 µg/ml groups (with and without metabolic activation) was not significantly increased above that of the solvent control. Under the conditions of this investigation, EGI was negative for the induction of structural and numerical chromosome aberrations in both the presence and absence of metabolic activation.

IV. High pI Xylanase from *T. reesei* (homologous rDNA)

A. 91-day subchronic feeding study in rats

This study was conducted in accordance with OECD Guideline 408 in CD rats. Groups of 10 male and female CD rats were fed with 4 different concentrations of the test material in the diet for 13 consecutive weeks (Pharmaco LSR, 1995). Two high dose rats (1 male and 1 female) died but the deaths were not attributed to be treatment related. There were no treatment-related adverse effects on any of the parameters (systemic toxicity, food consumption, opthalmology, clinical chemistry, body weight, organ weight, urine analysis, hematology, necropsy, and histopathology) monitored in this study for rats treated with *T. reesei* High pI xylanase. Based upon these findings, it was concluded that the treatment of male and female rats with High pI xylanase from *T. reesei* did not result in toxicity up to and including the highest dose tested in the diet. A NOEL was established at this dose.

B. Bacterial reverse mutation assay (Ames assay)

The test material, high pI xylanase from *T. reesei* was tested in four strains of *Salmonella typhimurium* (TA98, TA100, TA 1535 and TA1537) and *Escherichia coli* tester strain WP2 *uvr*A in the presence and absence of metabolic activation. The assay was conducted in accordance with OECD Guideline 471.

In the first assay, various dose levels ranging from 0 to 5,000 µg/plate were used and no positive mutagenic response was observed at any dose level in any of the exposure groups. In the confirmatory assay, dose levels up to 5,000 µg/plate were used. No positive mutagenic response was observed in both the presence and absence of metabolic activation. Under the conditions of this assay, high pI xylanase from *T. reesei* was not a mutagen.

C. *In vitro* chromosomal aberration assay with human peripheral blood lymphocytes

The test material, high pI xylanase from *T. reesei* was tested in the *in vitro* mammalian chromosome aberration test using human peripheral blood lymphocytes (HPBL) in both the absence and presence of metabolic activation. The assay was conducted in accordance with OECD Guideline 473.

In a preliminary toxicity assay, HPBL cells were exposed to various concentrations of the test material ranging from 0.5 to 5000 µg/ml and solvent controls in both the presence and absence of metabolic activation. Cytotoxicity was not observed at any dose level in any of the exposure groups. Based on these findings, dose levels up to 5000 µg/ml were selected for the chromosome aberration assay. In the main chromosome aberration assay, HPBL cells were incubated with the test material for 4 and 20 hours in the non-activated test system and for 4 hours in the activated test system. All cells were harvested 20 hours after treatment. Mitomycin C and cyclophosphamide served as positive controls. No statistically or biologically significant increases in the number of cells with aberrations (structural or numerical) were noted for the test material treated cells. Under the conditions of this investigation, high pI xylanase from *T. reesei* did not induce any clastogenic or aneugenic effects in cultured human lymphocytes, either with or without metabolic activation.

V. Endoglucanase II from *T. reesei* (homologous rDNA)

B. Bacterial Reverse Mutation Assay – Ames assay

The objective of this assay was to assess the potential of Endoglucanase II to induce point mutations (frame-shift and base-pair) in four strains of *Salmonella typhimurium* TA 98, TA 100, TA 1535 and TA 1537 and *Escherischia coli* strain WP2 uvr in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). This assay was conducted in accordance with OECD guideline No. 471 (1997), the Commission Regulation (EC) No. 440/2008 dated May 2008, and the US EPA (TSCA) OPPTS harmonized guidelines. The assay also complied with OECD Principles of GLP (1997) and all subsequent OECD consensus documents.

In the main test, five dose levels (50, 150, 500, 1500 and 5000 µg TP/plate) were tested. The plates incubated with the test material showed normal background growth up to 5000 µg/plate with and without metabolic activation. No biologically significant increases in the number of revertant colonies were observed at any dose level of the test item in either main test. There was also no tendency of higher mutation rates with increasing concentrations of the test material. 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 assay and the efficacy of the metabolic activation mixture.

Under the conditions of this assay, Endoglucanase II from *T. reesei* has not shown any evidence of mutagenic activity in the Ames assay. The glucanase did not induce gene mutations by base pair changes or frame-shifts in the genome of the strains used.

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

The objective of this assay was to investigate the potential of the endoglucanase II to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). This assay was conducted in accordance with OECD guideline No. 473 (1977) and the Commission Regulation (EC) No. 440/2008 dated May 2008. The assay complied with OECD Principles of GLP (1997) and all subsequent OECD consensus documents.

In the preliminary test, all cultures (with or without S-9 mix) were treated for 4 hours. In the main test, cultures without S-9 mix were treated for 22 hours and those with S-9 mix for 4 hours. Three hours before harvesting, colcemid was added to all cultures at the concentration of 0.2 µg/ml to arrest all cells at the metaphase stage of mitosis. All cultures (with and without S-9 mix) were harvested by centrifugation 22 hours after the start of treatment. The supernatant was discarded and the cell pellets were re-suspended in a KCl hypotonic solution. The cell suspension was allowed to stand at 37°C for 25 minutes and then centrifuged. The hypotonic solution was removed. The cells were then fixed on slides, stained and scored for chromosomal aberrations.

Under the conditions of this test, Endoglucanase II did not induce chromosomal aberrations (both structural and numerical) in this *in vitro* cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation up to the highest concentration (5000 µg TP/ml) recommended by guidelines.

C. 13-week Oral (Gavage) Toxicity Study in Wistar Rats

The objective of this study was to investigate the potential of Endoglucanase II to induce systemic toxicity after repeated daily oral (gavage) administration to Wistar rats of both sexes. This study was conducted in accordance with OECD guideline No. 408 (September 1998) and Directive 96/54/EC, B.26. "Subchronic Oral Toxicity", 30 September 1996 and in compliance with the OECD Principles of Good Laboratory Practice [November 1997] and the US EPA Health Effects Test Guidelines, OPPTS 870.3100.

Daily administration of Endoglucanase II by oral gavage to Wistar rats at4 different dosages for 13 consecutive weeks resulted in some effects in high dose males but were not considered as adverse. Indeed, centrilobular hepatocytes hypertrophy of the liver was noted in 3 out of 10 high dose males. However, hepatocyte enlargement is a common histopathologic finding in rodent liver and, in the absence of associated degenerative or inflammatory changes; this finding is generally considered to be an adaptive response. Both relative and absolute adrenal weights were increased in high dose males but this finding is a non-specific common stress response in rodents usually associated with increased steroidogenesis. In the absence of corresponding histopathologic changes in the adrenal glands, the toxicological significance of this finding is questionable. This

reviewer agrees with the conclusion from the study director and pathologist that the effects noted in high dose males were not considered to represent an adverse effect to health.

Under the conditions of this assay, the NOAEL was established at the highest dose tested

VI. Endoglucanase III from *T. reesei* (homologous rDNA)

A. 28-day oral study in rats

This study was conducted in accordance with OECD Guideline 408 in rats. Endoglucanase III was given by gavage to groups of male and female Charles River rats at 4 different concentrations for 28 consecutive days (Pharmakon, 1995).

No mortalities were recorded throughout the entire investigation. There were no treatment-related adverse effects on any of the parameters (systemic toxicity, food consumption, clinical chemistry, body weight, organ weight, urine analysis, hematology, necropsy, and histopathology) monitored in this study for rats treated with the enzyme. Based upon these findings, it was concluded that the treatment of male and female rats with EG III did not result in toxicity and a NOEL was established at the highest dosage tested.

B. Bacterial reverse mutation assay (Ames assay)

The test article was examined for mutagenic activity in 5 strains of *Salmonella typhimurium* and *Escherichia coli* strain WP2*uvr*A in both the presence and absence of metabolic activation (Hazleton, 1995). The assay was conducted in accordance with OECD Guideline 471.

In the first assay, various dose levels ranging from 2.5 to 5,000 µg/plate were used and no positive mutagenic response was observed at any dose level in any of the exposure groups. In the confirmatory assay, dose levels of 0, 333, 667, 1,000, 3,330, or 5,000 µg/plate were used. No positive mutagenic response was observed in both the presence and absence of metabolic activation. Under the conditions of this investigation, the enzyme was not mutagenic in the bacterial reverse mutation assay using *Salmonella* and *E. coli*.

VII. Low pI Xylanase from *T. reesei* (homologous rDNA)

A. 91-day subchronic oral study in rats

This study was conducted in accordance with OECD Guideline 408 in CD rats. Groups of male and female rats were treated orally with 4 concentrations of the test material for 13 consecutive weeks (BioResearch, 1997).

No mortalities were recorded throughout the entire investigation. There were no treatment-related adverse effects on any of the parameters (systemic toxicity, food consumption, opthalmology, clinical chemistry, body weight, organ weight, urine analysis, hematology, necropsy, and histopathology) monitored in this study for rats treated with Low pI xylanase from *T. reesei.* Based upon these findings, it was concluded that the treatment of male and female rats with low pI xylanase from *T. reesei* did not result in toxicity up to and including the highest dose tested. A NOEL was established at the highest dose rate.

B. Bacterial reverse mutation assay (Ames assay)

The test material, low pI xylanase from *T. reesei,* was tested in four strains of *Salmonella typhimurium* (TA98, TA100, TA 1535 and TA1537) and *Escherichia coli* tester strain WP2 *uvr*A in the presence and absence of metabolic activation (BioReliance, 1997). The assay was conducted in accordance with OECD Guideline 471.

In the first assay, eight dose levels ranging from 0 to 5,000 µg/plate were used and no positive mutagenic response was observed at any dose level in any of the exposure groups. In the confirmatory assay, dose levels up to 5,000 µg/plate were used. No positive mutagenic response was observed in both the presence and absence of metabolic activation. Under the conditions of this assay, low pI xylanase from *T. reesei* was not a mutagen up to the maximum recommended dose level of 5,000 µg/plate in both the presence and absence of metabolic activation.

C. *In vitro* chromosomal aberration assay with human peripheral blood lymphocytes

The test material, low pI xylanase from *T. reesei* was tested in the *in vitro* mammalian chromosome aberration test using human peripheral blood lymphocytes (HPBL) in both the absence and presence of metabolic activation (BioReliance, 1997). The assay was conducted in accordance with OECD Guideline 473.

In a preliminary toxicity assay, HPBL cells were exposed to 9 concentrations of the test material ranging from 0.5 to 5000 µg/ml and solvent controls in both the presence and absence of metabolic activation. Cytotoxicity was not observed at any dose level in any of the exposure groups. Based on these findings, dose levels up to 5000 µg/ml were selected for the chromosome aberration assay. In the main chromosome aberration assay, HPBL cells were incubated with the test material for 4 and 20 hours in the non-activated test system and for 4 hours in the activated test system. All cells were harvested 20 hours after treatment. Mitomycin C and cyclophosphamide served as positive controls. No statistically or biologically significant increases in the number of cells with aberrations (structural or numerical) were noted in the test material treated cells. Under the conditions of this investigation, low pI xylanase from *T. reesei* did not induce any clastogenic or aneugenic effects in cultured human lymphocytes, either with or without metabolic activation.

VIII. Xylanase from *T. reesei* (homologous rDNA)

A. 91-day subchronic oral study in rats

A 13-week oral gavage study with xylanase from *T. reesei* was conducted in accordance with OECD Guideline 408 in CD rats. Groups of ten male and females rats each were exposed to 3 different concentrations of the test material by gavage in a constant volume of 5 ml/kg/day. Control animals received deionized water following the same treatment regimen (MDS Pharma, 2002).

No mortalities were recorded throughout the entire investigation. There were no treatment-related adverse effects on any of the parameters (systemic toxicity, food consumption, opthalmology, clinical chemistry, body weight, organ weight, urine analysis, hematology, necropsy, and histopathology) monitored in this study for rats treated with xylanase from *T. reesei.* There were no differences in behavioral test (open field) and functional tests (gripping reflex, startle reflex) conducted at study termination between the control and treated groups. Based upon these findings, it was concluded that the treatment of male and female rats with *T. reesei* xylanase did not result in toxicity up to and including the highest dosage tested. A NOAEL was established at this concentration.

B. Bacterial reverse mutation assay (Ames assay)

The test material, xylanase *T. reesei* (homologous rDNA) was tested in four strains of *Salmonella typhimurium* (TA98, TA100, TA 1535 and TA1537) and *Escherichia coli* tester strain WP2 *uvr*A in the presence and absence of metabolic activation (MDS Pharma, 2002). The assay was conducted in accordance with OECD Guideline 471 using the plate incorporation method (first assay) and treat and plate method (second assay).

In the first assay, dose levels ranging from 52 to 5,000 µg/plate were used and no treatment-related positive mutagenic response was observed at any dose level in any of the exposure groups. In the second assay, dose levels ranging from 492 to 5,000 µg/plate were used. No precipitate or cytotoxicity was noted. Random changes in the number of revertants were noted in TA 98, TA 100 and TA 1535. However, in the absence of a dose response relationship, these changes were considered as spontaneous variations. Under the conditions of this assay, xylanase from *T. reesei* was not a mutagen in both the presence and absence of metabolic activation.

C. *In vitro* chromosomal aberration assay with human peripheral blood lymphocytes

The test material, a xylanase from *T. reesei,* was tested in the *in vitro* mammalian chromosome aberration test using human peripheral blood lymphocytes (HPBL) in both the absence and presence of metabolic activation (MDS Pharma, 2003). The assay was conducted in accordance with OECD Guideline 473.

In a preliminary toxicity assay, HPBL cells were exposed to various concentrations of the test material ranging from 0.0 to 3600 µg/ml and solvent controls in both the presence and absence of metabolic activation. Cytotoxicity was not observed at any dose level in any of the exposure groups. Based on these findings, dose levels up to 3600 µg/ml were selected for the chromosome aberration assay. In the chromosome aberration assay, HPBL cells were incubated with the test material for 4 and 20 hours in the non-activated test system and for 4 hours in the activated test system. All cells were harvested 20 hours after treatment. Two hours prior to harvest, Demecolcine (0.1 µg/ml) was added to all cultures to arrest all cells at the metaphase-stage of mitosis. Mitomycin C and cyclophosphamide served as positive controls. No statistically or biologically significant increases in the number of cells with aberrations (structural or numerical) were noted for the test material treated cells. Under the conditions of this investigation, xylanase from *T. reesei* did not induce any clastogenic or aneugenic effects in cultured human lymphocytes, either with or without metabolic activation.

IX. Protease from *T. reesei* (homologous rDNA)

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

The objective of this study was to investigate the potential of protease from *T. reesei* to induce systemic toxicity after repeated daily oral administration to SPF Sprague Dawley rats (Taconic M&B, Denmark) of both sexes for 90 consecutive days.
This study was conducted in accordance with OECD guideline No. 408 (September 1998) and EPA Guideline OPPTS 870.3100 (August 1998) and complied with OECD Principles of GLP (as revised in 1997) and all subsequent OECD consensus documents.

There were no treatment-related deaths in this study. No clinical signs were seen that could be considered to be treatment related. There were no biological or statistical differences between the control and treated groups with respect to feed consumption, body weights, body weight gains, hematology, clinical chemistry, and ophthalmologic examinations. At study termination, in the males, the absolute and relative liver weights were statistically significantly increased and the relative testes weight was statistically significantly decreased compared to the concurrent control group. However, there were no treatment-related macroscopic and histopathologic changes. In the functional observation battery testing, there were no statistically significant changes noted in treated groups.

Although statistically significant variations in liver and testes weights were noted in high dose males, these weight variations were still within the 95% confidence interval of historical control data collected at LAB Scantox. Further, in the absence of corresponding histopathologic changes, these weight variations were considered to be of no toxicological significance. Under the conditions of this assay, the NOAEL is established at the highest dose tested.

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

The objective of this assay is to investigate the potential of protease from *T. reesei* to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). This assay was conducted in accordance with OECD guideline No. 473 (*In vitro* Mammalian chromosome aberration test) and complied with OECD Principles of GLP (as revised in 1997) and all subsequent OECD consensus documents.

No biologically or statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with AFP concentrate both in the presence and absence of metabolic activation. Significant increases in aberrant metaphases were demonstrated with the positive controls. Under the conditions of this test, the enzyme concentrate did not induce chromosomal aberrations (both structural and numerical) in mammalian cells both in the presence and absence of metabolic activation.

C. Bacterial Reverse Mutation Assay – Ames assay

The objective of this assay is to assess the potential of protease from *T. reesei* 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) in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). This assay was conducted in accordance with OECD guideline No. 471 and complied with OECD Principles on GLP (as revised in 1997) and all subsequent OECD consensus documents.

Although a statistically significant reduction in the number of revertant colonies was noted in TA 1535 with S-9 mix, this is not considered to be biologically significant in the absence of a dose-related effect. Some variations in revertant colonies were noted but the variations were not reproducible between the three replicate plates and none of these variations meet the positive criteria recognized by regulatory agencies worldwide. Under the conditions of this assay, the test article has not shown any evidence of mutagenic activity in the Ames assay.

X. Phosphatase (4-Phytase) from *T. reesei* (heterologous rDNA)

A. Bacterial Reverse Mutation Assay – Ames assay

The objective of this assay was to assess the potential of phosphatase (4-phytase) to induce point mutations (frame-shift and base-pair) in five strains of *Salmonella typhimurium*(TA 98, TA 100, TA 102, TA 1535 and TA 1537) both in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). This assay was conducted in accordance with OECD guideline No. 471 and complied with OECD Principles on GLP (as revised in 1997) and all subsequent OECD consensus

documents. Under the conditions of this assay, phosphatase (4-phytase) from *T. reesei* has not shown any evidence of mutagenic activity in the Ames assay.

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

The test material, a phosphatase from *T. reesei,* was tested *in vitro* in the chromosome of mammalian systems (i.e., human peripheral lymphocytes) both in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver; S-9 mix). This assay was conducted in accordance with OECD guideline No. 473 (*In vitro* Mammalian chromosome aberration test) and complied with OECD Principles of GLP (as revised in 1997) and all subsequent OECD consensus documents.

In the presence of S-9 mix, no biologically or statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with Phosphatase (4-phytase).

In the absence of S-9 mix, statistically significant increases were observed at three test points, but none of these increases are considered to be biologically significant. Significant increases in aberrant metaphases were demonstrated with the positive controls. Under the conditions of this test, Phosphatase (4-phytase) did not induce chromosomal aberrations (both structural and numerical) in this *in* vitro cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation.

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

The objective of this study was to investigate the potential of Phosphatase (4-phytase) to induce systemic toxicity after repeated daily oral administration to SPF Sprague Dawley rats (Taconic M&B, Denmark) of both sexes for 90 consecutive days. This study was conducted in accordance with OECD guideline No. 408 (September 1998) and EPA Guideline OPPTS 870.3100 (August 1998) and complied with OECD Principles of GLP (as revised in 1997) and all subsequent OECD consensus documents.

No mortalities were recorded throughout the entire investigation. There were no overt signs of systemic toxicity or clinical observations that could be considered treatment-related. There were no biological or statistical differences between the control and treated groups with respect to feed consumption, water consumption, body weights, body weight gains, hematology, and ophthalmologic examinations.

Daily administration of Phosphatase (4-phytase) by gavage for 90 consecutive days did not result in adverse systemic toxicity or adverse effects on clinical chemistry, hematology, functional observation tests and macroscopic and histopathologic examinations. Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established at the highest dose tested.

XI. Chymosin

A. Bacterial Reverse Mutation Assay – Ames assay

The test material, chymosin from *T. reesei,* was tested in five strains of *Salmonella typhimurium*(TA 98, TA 100, TA 102, TA 1535 and TA 1537) in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). This assay was conducted in accordance with OECD guideline No. 471 and complied with OECD Principles on GLP (as revised in 1997) and all subsequent OECD consensus documents.

In the screening assay, chymosin was not toxic to the test bacteria except at the highest dose level (5000 µg/plate) in the presence of S-9 mix where the growth of the background lawn was slightly reduced. Therefore, 5000 µg/plate was selected as the highest dose level for the main tests. In the main tests, five dose levels (50, 160, 500, 1600 and 5000 µg/plate) were tested. Insoluble material was observed on most of the plates treated at all dose levels. Slight reductions in the growth of the background lawn were observed in strains TA 98 and TA 1537 in the presence of S-9 mix. A statistically significant reduction in the number of revertant colonies wwas observed in the second main test with TA 1537 in the absence of S-9 mix.

No biologically significant increases in the number of revertant colonies were observed in any dose level in either main test. A statistically significant increase in the number of revertant colonies were observed in the first main test with TA 1537 at the lowest dose tested, 50 µg/plate, in the absence of S-9 mix and at the 500 µg/plate in the presence of S-9 mix. These increases were not considered as treatment-related as they are not dose-related and are found only in the first 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. Under the conditions of this assay, Chymosin has not shown any evidence of mutagenic activity in the Ames assay.

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

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

This assay was conducted in accordance with OECD guideline No. 473 (*In vitro* Mammalian chromosome aberration test) and complied with OECD Principles of GLP (as revised in 1997) and all subsequent OECD consensus documents.
In both the presence and absence of S-9 mix, no biologically or statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with chymosin. Significant increases in aberrant metaphases were

demonstrated with the positive controls demonstrating the sensitivity of the tests and the efficacy of the S-9 mix.Under the conditions of this test, Chymosin did not induce chromosomal aberrations (both structural and numerical) in this *in* vitro cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation.

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

The objective of this study was to investigate the potential of Chymosin to induce systemic toxicity after repeated daily oral administration to SPF Sprague Dawley rats (Taconic M&B, Denmark) of both sexes for 90 consecutive days. This study was conducted in accordance with OECD guideline No. 408 (September 1998) and EPA Guideline OPPTS 870.3100 (August 1998) and complied with OECD Principles of GLP (as revised in 1997) and all subsequent OECD consensus documents.

One mid-dose female (# 51) was found dead on day 13 and necropsied. Since no clinical signs were observed in this animal prior to death, the cause of death was not related to treatment. No mortalities were recorded in other groups throughout the entire investigation. There were no overt signs of systemic toxicity or clinical observations that could be considered treatment-related. There were no biological or statistical differences between the control and treated groups with respect to feed consumption, water consumption, body weights, body weight gains, hematology, and ophthalmologic examinations. On day 90, the "Total Distance Parameter" in the open-field test was statistically significantly higher for high dose males. All other parameters of the Functional Observation Battery Test were not significantly different from controls. The biological significance of this finding was not clear since the significant value was still within the range of the historical control data collected at the testing facility.

Incidental macroscopic findings were noted but were not considered as treament-related. Scattered histopathologic findings were noted, but in the abscense of a dose-response relationship all findings were considered to be within the background incidence of findings reported in this age and strain of laboratory animals.

Daily administration of Chymosin by gavage for 90 consecutive days did not result in adverse systemic toxicity or adverse effects on clinical chemistry, hematology, functional observation tests and macroscopic and histopathologic examinations. Under the conditions of this assay, the NOAEL was established at the highest dose tested.

XII. Transglucosidase from *T. reesei* (heterologous rDNA)

A. Bacterial Reverse Mutation Assay – Ames assay

The objective of this assay was to assess the potential of transglucosidase from *T. reesei* to induce point mutations (frame-shift and base-pair) in four strains of *Salmonella*

typhimurium TA 98, TA 100, TA 1535 and TA 1537 and *Escherischia coli* strain WP2 uvrA in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). This assay was conducted in accordance with OECD guideline No. 471 (1997), the Commission Regulation (EC) No. 440/2008 dated May 2008 and complied with OECD Principles of GLP (1997) and all subsequent OECD consensus documents.

In the screening assay, the transglucosidase was not toxic to the test bacteria up to and including the highest dose level (5000 µg TP/plate) in both absence and presence of S-9 mix. Therefore, 5000 µg TP/plate was selected as the highest dose level for the main test. In the main test, five dose levels (33, 100, 333, 1000, 2500 and 5000 µg TP/plate) were tested. The plates incubated with the test material showed normal background growth up to 5000 µg/plate with and without metabolic activation. No biologically significant increases in the number of revertant colonies were observed at any dose level of the test item in either main test. There was also no tendency of higher mutation rates with increasing concentrations of the test material. Statistical increases in the number of revertant colonies were noted with the positive controls in both the presence and absence of metabolic activation substantiating the sensitivity of the treat and plate assay and the efficacy of the metabolic activation mixture.

Under the conditions of this assay, transglucosidase from *T. reesei* has not shown any evidence of mutagenic activity in the Ames assay. The test material did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

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

The objective of this assay was to investigate the potential of transglucosidase from *T. reesei* to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). This assay was conducted in accordance with OECD guideline No. 473 (1977), the Commission Regulation (EC) No. 440/2008 dated May 2008 and complied with OECD Principles of GLP (1997) and all subsequent OECD consensus documents.

Preliminary assay (Experiment I): Ten dose levels were used. Exposure period was 4 hours for both cultures with and without S9 mix. No clear cytotoxicity was observed up to highest concentration tested 5000 µg TP/ml. No visible precipitation of the test material in the culture medium was observed. No biologically relevant increases in cells with chromosomal aberrations were noted in the three highest dose levels selected for analysis. Since the cultures fulfilled the requirements for cytogenicity evaluation, this preliminary assay was designated as Experiment I.

In experiment II: Exposure period was 4 hours for cultures with S9 mix and 22 hours for cultures without S9 mix. No visible precipitation of the test material in the culture medium was observed. In the absence of S9 mix (22-hour cultures), mitotic index was

reduced to 53.85 of control after treatment with 5000 µg/ml. In the presence of S9 mix (4-hour cultures), no clear cytotoxicity was observed up to the highest concentration tested, 5000 µg/ml. No biologically relevant increases in cells with chromosomal aberrations were noted in the three highest dose levels selected for analysis. (1632.7, 2857.1 and 5000 µg TP/ml).

Under the conditions of this test, the test article did not induce chromosomal aberrations (both structural and numerical) in this *in vitro* cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation up to the highest concentration (5000 µg TP/ml) recommended by guidelines.

C. 18-week Oral (Gavage) Toxicity Study in Wistar Rats

The objective of this study was to investigate the potential of transglucosidase from *T. reesei* to induce systemic toxicity after repeated daily oral administration to SPF-bred Wistar rats of both sexes. This study lasted 18 weeks and consisted of 2 phases. During the first phase (5-week duration), groups of 10 animals per sex were treated by oral gavage daily with 4 different concentrations. Due to the absence of overt signs of toxicity in phase 1, both study director and study sponsor agreed to increase the dose levels. The higher dose levels were administered by oral gavage to the animals for an additional 13-week period (phase 2). All animals were sacrificed at termination of phase 2 (week 18 of study).

This study was conducted in accordance with OECD guideline No. 408 (September 1998) and Directive 96/54/EC, B.26. "Subchronic Oral Toxicity", 30 September 1996 and in compliance with the Swiss Ordinance relating to Good Laboratory Practice [May 18, 2005] and the OECD Principles of Good Laboratory Practice [November 1997].

Daily administration of transglucosidase by oral gavage to Wistar rats at lower doses for a 5-week period and at higher doses for an additional 13-week period resulted in no treatment-related deaths, clinical observations, feed consumption, body weight changes, hematology, clinical chemistry, urinalysis, organ weights, functional observation, grip strength and locomotor activities. No macroscopic or microscopic changes could be attributed to treatment. Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established at the highest dose tested.

XIII. Glucoamylase from *T. reesei* (homologous rDNA)

A. Bacterial Reverse Mutation Assay – Ames assay

The objective of this assay was to assess the potential of glucoamylase from *T. reesei* (TrGA) to induce point mutations (frame-shift and base-pair) in five strains of *Salmonella typhimurium* (TA 98, TA 100, TA 102, TA 1535 and TA 1537) in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix. This assay was conducted in accordance with OECD guideline No. 471 and complied with

OECD Principles on GLP (as revised in 1997) and all subsequent OECD consensus documents.

In the screening assay, TrGA was not toxic to the test bacteria up to and including the highest dose level (5000 µg/plate) in both absence and presence of S-9 mix. In the main tests, five dose levels (50, 160, 500, 1600 and 5000 µg/plate) were tested. No marked reductions in the number of revertant colonies or growth of the background lawn of non-revertant bacteria were observed. No biologically significant increases in the number of revertant colonies were observed at any dose level of the test item in either main test. Small, statistically significant increases in the number of revertant colonies were observed at several test points, but none are considered to be biologically significant (i.e., at least a 2-fold higher than the corresponding negative control value) since the increases were not clearly dose-related and were not reproducible.Statistical increases in the number of revertant colonies were noted with the positive controls in both the presence and absence of metabolic activation substantiating the sensitivity of the treat and plate assay and the efficacy of the metabolic activation mixture. Under the conditions of this assay, TrGA has not shown any evidence of mutagenic activity in the Ames assay.

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

The objective of this assay was to investigate the potential of TrGA to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). This assay was conducted in accordance with OECD guideline No. 473 (*In vitro* Mammalian chromosome aberration test) and complied with OECD Principles of GLP (as revised in 1997) and all subsequent OECD consensus documents.

In both the presence and absence of S-9 mix, no biologically or statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with TrGA in either test. Significant increases in aberrant metaphases were demonstrated with the positive controls demonstrating the sensitivity of the tests and the efficacy of the S-9 mix. Under the conditions of this test, TrGA did not induce chromosomal aberrations (both structural and numerical) in this *in vitro* cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation.

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

The objective of this study was to investigate the potential of TrGA to induce systemic toxicity after repeated daily oral administration to SPF Sprague Dawley rats (Taconic M&B, Denmark) of both sexes for 90 consecutive days. This study was conducted in accordance with OECD guideline No. 408 (September 1998) and EPA Guideline OPPTS 870.3100 (August 1998) and complied with OECD Principles of GLP (as revised in 1997) and all subsequent OECD consensus documents.

There were no biological or statistical differences between the control and treated groups with respect to feed consumption, water consumption, body weights, body weight gains, hematology, and ophthalmologic examinations. There were no differences in the functional observation battery and stimuli assays between treated and control animals. At study termination, the mean cell hemoglobin in high dose females was statistically lower than control values and the mean cell hemoglobin concentration in mid dose females was statistically significantly higher than control values, but these findings were considered incidental in the absence of a dose-response relationship and the observation of finding in only one gender. Changes in creatinine levels were also observed in treated females but all values were within the historical control range and were not considered as treatment-related.

At necropsy, there were no treatment related findings on organ weights, macroscopic findings and histopathologic examinations. In males, focal tubular basophilia/dilatation including hyaline casts in the kidneys and minimal testicular atrophy were noted in both control and high dose groups. In light of these findings, microscopic examination was extended to the kidneys and testes of males in the low and mid dose groups. Upon examination of all groups, the findings in the kidneys were considered incidental in the absence of a dose response relationship (the most severe findings were noted in mid dose group) and no statistical differences were detected among groups. Further, the incidences of focal tubular basophilia/dilatation with hyaline casts in this study were within the range of historical control data for this species and strain. Testicular atrophy (minimal severity) was noted in all groups, including the control, and was considered as incidental and of no toxicological significance.

All other microscopic findings were considered to be within the background incidence of findings reported in this age and strain of laboratory animals.
Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established at the highest dose tested.

XIV. Lipase from *T. reesei* (heterologous rDNA)

A. Bacterial Reverse Mutation Assay – Ames assay

The objective of this assay was to assess the potential of lipase from *T. reesei* to induce point mutations (frame-shift and base-pair) in four strains of *Salmonella typhimurium* TA 98, TA 100, TA 1535 and TA 1537 and *Escherischia coli* strain WP2 uvrA in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). This assay was conducted in accordance with OECD guideline No. 471 (July 21, 1997), EPA OPPTS 870.5100 (August 1998) and complied with OECD Principles on GLP (as revised in 1997) and all subsequent OECD consensus documents.

The plates incubated with the test material showed normal background growth up to 5000 µg/plate with and without metabolic activation. No biologically significant increases in the number of revertant colonies were observed at any dose level of the test item. There

was also no tendency of higher mutation rates with increasing concentrations of the test material. Statistical increases in the number of revertant colonies were noted with the positive controls in both the presence and absence of metabolic activation substantiating the sensitivity of the treat and plate assay and the efficacy of the metabolic activation mixture. Under the conditions of this assay, the test article has not shown any evidence of mutagenic activity in the Ames assay; it did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

B. *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes (Harlan Laboratories)

The objective of this assay was to investigate the potential lipase from *T. reesei* to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). This assay was conducted in accordance with OECD guideline No. 473 (*In vitro* Mammalian chromosome aberration test; February 1998) and complied with Commission Regulation (EC) No. 440/2008 B.10: "Mutagenicity – In Vitro Mammalian Chromosome Aberration Test" dated May 30, 2008. The study was performed in compliance with the Chemicals Act of the Federal Republic of Germany (July 25, 1994; revised June 27, 2002) and the OECD Principles of Good Laboratory Practice (1997).

In both the preliminary and main assays, no increase in polyploidy metaphases was noticed. Under the conditions of this test, the test article did not induce chromosomal aberrations (both structural and numerical) in this *in vitro* cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation up to the highest concentration (5000 µg/ml) recommended by guidelines.

C. 13-week Oral (Gavage) Toxicity Study in Wistar Rats

The objective of this study was to investigate the potential of lipase from *T. reesei* to induce systemic toxicity after repeated daily oral administration (gavage) to SPF-bred Wistar rats of both sexes. This study was conducted in accordance with OECD guideline No. 408 (September 1998) and Directive 96/54/EC, B.26. "Subchronic Oral Toxicity", 30 September 1996 and in compliance with the Swiss Ordinance relating to Good Laboratory Practice (May 18, 2005) and the OECD Principles of Good Laboratory Practice (1997).

Daily administration of the test article for 13 consecutive weeks by oral gavage to Wistar rats daily at 4 different concentrations resulted in no treatment-related deaths, clinical observations, feed consumption, body weight changes, hematology, clinical chemistry, urinalysis, organ weights, functional observation, grip strength and locomotor activities. No macroscopic or microscopic changes could be attributed to treatment. Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established at the highest dose tested.

XV. Alpha-amylase from *T. reesei* (heterologous rDNA)

A. Bacterial Reverse Mutation (Ames) Assay

The objective of this assay was to investigate the mutagenic potential of alpha-amylase from *T. reesei* using *Salmonella typhimurium* strains TA 102, TA 100, TA 98, TA 1537, and TA 1535. This assay was conducted in accordance with OECD guideline No. 471 (1997), the Commission Regulation (EC) No. 440/2008 dated May 2008 and complied with OECD Principles of GLP (1997) and all subsequent OECD consensus documents.

The test item was not toxic to any tester strain at any dose level with or without metabolic activation. No biologically significant increases were seen in any tester strain at any dose level with or without metabolic activation. The positive controls used in the test yielded marked increases in the frequency of revertant colonies, confirming the efficacy of the S9 mix and the sensitivity of the bacterial strains.
Under the conditions of this assay, the test article was found to be non-mutagenic in the Ames test.

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

The objective of this procedure was to investigate the potential of alpha-amylase from *T. reesei* to induce numerical and/or structural changes in mammalian chromosomes (human peripheral lymphocytes). This assay was conducted in accordance with OECD guideline No. 473 (1997), the Commission Regulation (EC) No. 440/2008 dated May 2008 and complied with OECD Principles of GLP (1997) and all subsequent OECD consensus documents.

The test item did not cause marked toxicity at any concentration tested in the presence or absence of S-9 mix in any test. In the initial test 1, exposure to the alpha-amylase with metabolic activation at 5000 µg total protein/ml produced a small but statistically significant increase in the mean frequency of aberrant metaphases. This result was not reproducible in the replicate culture so it was further investigated in the repeated test 1. In the initial test 2, three of the four positive control treatments did not produce adequate increases in the frequency of aberrant metaphases. Exposure to 1250 µg total protein/ml without S-9 mix yielded a statistically significant increase in the mean frequency of aberrant metaphases. However, this result was not reproducible between the replicate cultures and it was further investigated in the repeated test 2. No biologically or statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with the alpha-amylase in the presence or absence of S-9 mix in the repeated test 1. The repeated test 2 was considered valid since all controls performed as expected. No biologically or statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with the alpha-amylase in the presence or absence of S-9 mix in the repeated test 2. The increases in frequency of aberrant metaphases in both initial tests 1 and 2 are not

considered to be biologically significant since they were not reproduced in the replicate cultures or in the repeated tests. Under the conditions of this assay, the test article did not induce chromosomal aberrations (both structural and numerical) in this *in-vitro* test using human peripheral lymphocytes both in the presence and absence of metabolic activation up to the highest dose level (5000 µg total protein/ml) as recommended by the guidelines. The alpha-amylase is neither a clastogen nor an aneugen.

C. 90-day Oral Gavage Study in Rats

The objective of this study was to investigate the potential of alpha-amylase from *T. reesei* to induce systemic toxicity after repeated daily oral administration by gavage to Sprague Dawley rats of both sexes for 13 consecutive weeks. This study was conducted in accordance with OECD guideline No. 408 (September 1998), EU Annex B method B26 "Subchronic Oral Toxicity Test- Repeated Dose 90-Day Oral Toxicity Study in Rodents," 21 August 2001, USEPA Health Effects Test Guidelines OPPTS 870.3100 and in compliance with the Swiss Ordinance relating to Good Laboratory Practice [May 18, 2005] and the OECD Principles of Good Laboratory Practice [November 1997].

No adverse clinical signs were observed that could be related to the test item. No observations were noted with regards to the weekly assessment. No adverse effects attributed to the test item were observed during the functional battery before termination of treatment. All animals gained weight during the study, and no effects on food or water consumption were observed. No abnormalities were noted upon ophthalmoscopic examination. No effects in hematology, clinical chemistry and organ weights were noted that were considered toxicologically significant. No abnormalities in necropsy and microscopic examinations were seen.

Oral administration of the alpha-amylase to rats at 4 different doses did not result in any toxicologically significant effects. The NOAEL ("No Observed Adverse Effect Level") was established at the highest dosage tested.

XVI. Cellulase, beta-glucosidase, and hemicellulases from *T. reesei* (heterologous rDNA)

A. Bacterial Reverse Mutation (Ames) Assay

The objective of this assay was to assess the potential of cellulase, beta-glucosidase, and hemicellulases from *T. reesei* to induce point mutations (frame-shift and base-pair) in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and *E.coli* strain WP2 *uvr*A- in the presence and absence of a metabolic activation system (rat liver homogenate metabolizing system, S9). This assay was conducted in accordance with OECD guideline No. 471 (1997), the Commission Regulation (EC) No. 440/2008 dated May 2008 and complied with OECD Principles of GLP (1997) and all subsequent OECD consensus documents.

In the preliminary test, the material was not found to be toxic to the tester strains. In the two main tests, no test material precipitate was observed on the plates at any of the doses tested in either the presence or absence of S9 mix. No significant increases in the frequency of revertant colonies were observed for any of the bacterial strains, at any dose level, either with or without S9 or exposure method. The positive controls used in the test yielded marked increases in the frequency of revertant colonies, confirming the efficacy of the S9 mix and the sensitivity of the bacterial strains.
Under the conditions of this assay, the test article was found to be non-mutagenic in the Ames assay.

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

The objective of this assay was to investigate the potential of the test article to induce numerical and/or structural changes in mammalian chromosomes of mammalian systems (human peripheral lymphocytes). This assay was conducted in accordance with OECD guideline No. 473 (1997), the Commission Regulation (EC) No. 440/2008 dated May 2008 and complied with OECD Principles of GLP (1997) and all subsequent OECD consensus documents.

In Experiment 1, evaluation of the slides determined there was no apparent toxicity, and at the highest dose level there were metaphases suitable for scoring in both exposure groups. The test material did not induce any statistically significant increases in the frequency of cells with aberrations either with or without S9-mix, nor did it induce a statistically significant increase in the numbers of polyploidy cells at any dose level in either exposure group.

In Experiment 2, assessment of the slides determined metaphases suitable for scoring were present at the highest dose level (5000 µg/ml) in both exposure groups (with and without S9-mix). The test material did not induce any statistically significant increases in the frequency of cells with aberrations either with or without S9-mix, nor did it induce a statistically significant increase in the numbers of polyploidy cells at any dose level in either exposure group. Under the conditions of this assay, the test article did not induce chromosomal aberrations (both structural and numerical) in this *in vitro* test using human peripheral lymphocytes both in the presence and absence of metabolic activation up to the highest dose level (5000 µg/ml) as recommended by the guidelines. The test article is neither a clastogen or an aneugen.

C. 90-day Oral Gavage Study in Rats

The objective of this study was to investigate the potential of the test article to induce systemic toxicity after repeated daily oral administration by gavage to Wistar rats of both sexes for 90 consecutive days. This study was conducted in accordance with OECD guideline No. 408 (September 1998), EU Annex B method B26 "Subchronic Oral Toxicity Test- Repeated Dose 90-Day Oral Toxicity Study in Rodents," 21 August 2001, USEPA Health Effects Test Guidelines OPPTS 870.3100 and in compliance with the

Swiss Ordinance relating to Good Laboratory Practice [May 18, 2005] and the OECD Principles of Good Laboratory Practice [November 1997].

There were no unscheduled deaths. No clinical signs of toxicity and no changes in behavior were observed. No functional performance changes were detected, and no treatment-related changes in sensory reactivity were observed. No adverse effects on body weights were recorded. No adverse changes in body weight, food and water consumption, ophthalmoscopy, hematology, blood chemistry and organ weights were noted. No abnormalities in necropsy and histopathology were seen.

Oral administration of the test article to rats daily at 4 different doses did not result in any toxicologically significant effects. The NOAEL was established at the highest dose tested.

XVII. Glucoamylase from *T. reesei* (heterologous rDNA)

A. Bacterial Reverse Mutation (Ames) Assay

The objective of this assay was to assess the potential of glucoamylase from *T. reesei* to induce point mutations (frame-shift and base-pair) in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and *E.coli* strain WP2 *uvr*A- in the presence and absence of a metabolic activation system (rat liver homogenate metabolizing system, S9). This assay was conducted in accordance with OECD guideline No. 471 (1997), the Commission Regulation (EC) No. 440/2008 dated May 2008 and complied with OECD Principles of GLP (1997) and all subsequent OECD consensus documents.

In the preliminary test, the material was not found to be toxic to the tester strains. In the two main tests, no biologically significant increases in the frequency of revertant colonies were observed for any of the bacterial strains, at any dose level, either with or without S9 or exposure method. The positive controls used in the test yielded marked increases in the frequency of revertant colonies, confirming the efficacy of the S9 mix and the sensitivity of the bacterial strains.
Under the conditions of this assay, the test article was found to be non-mutagenic in the Ames assay.

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

The objective of this assay was to investigate the potential of the test article to induce numerical and/or structural changes in mammalian chromosomes of mammalian systems (human peripheral lymphocytes). This assay was conducted in accordance with OECD guideline No. 473 (1997), the Commission Regulation (EC) No. 440/2008 dated May 2008 and complied with OECD Principles of GLP (1997) and all subsequent OECD consensus documents.

In Experiment 1, no clear cytotoxicity was observed up to the highest concentration tested and no visible precipitation of the test material in the culture medium was observed. No biologically relevant increases in cells with chromosomal aberrations were noted at any of the doses tested. The test article did not induce any statistically significant increases in the frequency of cells with aberrations in either the presence or absence of S-9 mix.

In Experiment 2, no biologically relevant increases in cells with chromosomal aberrations were noted at any of the doses tested. The test article did not induce any statistically significant increases in the frequency of cells with aberrations in either the presence or absence of S-9 mix. In both experiments, no increase in polyploidy metaphases was noticed. Additionally, in both experiments, significant increases in aberrant metaphases were demonstrated with the positive controls, demonstrating the sensitivity of the tests and the efficacy of the S-9 mix. Under the conditions of this assay, the test article did not induce chromosomal aberrations (both structural and numerical) in this *in vitro* test using human peripheral lymphocytes both in the presence and absence of metabolic activation up to the highest dose level (5000 µg/ml) as recommended by the guidelines. The test article is neither a clastogen nor an aneugen.

C. 90-day Oral Gavage Study in Rats

The objective of this study was to investigate the potential of the test article to induce systemic toxicity after repeated daily oral administration by gavage to Wistar rats of both sexes for 90 consecutive days. This study was conducted in accordance with OECD guideline No. 408 (September 1998) and Directive 96/54/EC, B.26. "Subchronic Oral Toxicity", 30 September 1996 and in compliance with the Swiss Ordinance relating to Good Laboratory Practice [May 18, 2005] and the OECD Principles of Good Laboratory Practice [November 1997].

There were no unscheduled deaths. No clinical signs of toxicity and no changes in behavior were observed. No functional performance changes were detected, and no treatment-related changes in sensory reactivity were observed. No adverse effects on body weights were recorded. No adverse changes in body weight, food and water consumption, ophthalmoscopy, hematology, blood chemistry and organ weights were noted. No abnormalities in necropsy were seen, nor any treatment-related findings on histopathologic examinations.

Oral administration of the test article to rats daily at 4 different doses did not result in any toxicologically significant effects. The NOAEL was thus established at the highest dose tested.

XVIII. Xylanase from *T. reesei* (heterologous rDNA)

A. Bacterial Reverse Mutation (Ames)

In a bacterial reverse mutation assay, no biologically significant increases in the number of revertant colonies were observed at any dose level in the presence and absence of metabolic activation up to the maximum recommended dose. Under the conditions of this assay, the test article is not a mutagen.

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

In an *in vitro* assay with human peripheral lymphocytes, the test article did not induce chromosomal aberrations (both structural and numerical) in this cytogenetic test in both the presence and absence of metabolic activation up to the maximum recommended dose. Under the conditions of this assay, the test article is not a clastogen.

C. 90-day Oral Gavage Study in Rats

In a 90-day oral (gavage) study, rats were gavaged daily with various doses of TOS/kg body weight in a constant volume of 5 ml/kg body weight. There were no biological or statistical differences between the control and treated groups with respect to feed consumption, water consumption, body weights, body weight gains, hematology, and ophthalmologic examinations. There were no differences in the functional observation battery and stimuli assays between treated and control animals. At necropsy, there were no treatment related findings on organ weights and macroscopic findings. Under the conditions of this assay, the NOAEL (no observed adverse effect level) was established at the highest dose tested.

XIX Catalase from *T. reesei* (heterologous rDNA)

A. Bacterial Reverse Mutation (Ames)

The objective of this assay was to assess the potential of Catalase to induce point mutations in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and *Escherischia coli* strain WP2 uvrA. The test material was tested both in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix).

Catalase was not toxic to the test bacteria up to and including the highest dose level tested in both the absence and presence of S-9 mix. No positive mutagenic responses were observed with any of the tester strains in the presence of S-9 mix or with tester strains TA1535, TA1537 and WP2 *uvr*A in the absence of S-9 mix.

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

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

The test article did not induce any statistically significant increases in the frequency of cells with aberrations in either the presence or absence of S-9 mix. No increase in polyploidy metaphases was noticed. Significant increases in aberrant metaphases were demonstrated with the positive controls demonstrating the sensitivity of the tests and the efficacy of the S-9 mix.

C. 90-day Oral Gavage Study in Rats

The objective of this study was to investigate the potential of TCT Catalase to induce systemic toxicity after repeated daily oral administration to CD rats of both sexes for 90 continuous days.

There were no biological or statistical differences between the control and treated groups with respect to clinical observation, feed consumption, ophthalmologic examinations, body weights, and body weight gains. There were no treatment-related changes in hematology and clinical chemistry at the end of week 13. There were no differences in the functional observation battery, grip strength and locomotor activity assays between treated and control animals.

At necropsy, there were no treatment related findings on organ weights, macroscopic findings and histopathologic examinations. All microscopic findings were considered to be within the background incidence of findings reported in this age and strain of laboratory animals.

XX Glucoamylase from *T. reesei* (heterologous rDNA)

A. Bacterial Reverse Mutation (Ames)

The objective of this assay was to assess the potential of glucoamylase to induce point mutation in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and *Escherischia coli* strain WP2 uvrA. The test material was tested both in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). The assay was performed in two phases using the plate incorporation methodology for the positive control, 2-aminoanthracene, with *E. coli* and the treat and plate methodology for the all remaining strains and assays. This assay was conducted in accordance with OECD guideline No. 471 (1997).

In the screening assay, the glucoamylase was not toxic to all four *Salmonella* strains up to and including the highest dose level tested in both the absence and presence of S-9 mix. The assay was retested and in the repeat assay no toxicity was noted. No positive mutagenic responses were observed with any of the tester strains in the presence and absence of S-9mix.

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

The objective of this assay was to investigate the potential of the glucoamylase to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). This assay was conducted in accordance with OECD guideline No. 473 (1997), the Commission Regulation (EC) No. 440/2008 dated May 2008 and complied with OECD Principles of GLP (1997) and all subsequent OECD consensus documents.

The test article did not induce any statistically significant increases in the frequency of cells with aberrations in either the presence or absence of S-9 mix. No increase in polyploidy metaphases was noticed. Significant increases in aberrant metaphases were demonstrated with the positive controls demonstrating the sensitivity of the tests and the efficacy of the S-9 mix.

C. 90-day Oral Gavage Study in Rats

The objective of this study was to investigate the potential of the glucoamylase to induce systemic toxicity after repeated daily oral administration to rats of both sexes for 90 continuous days.

There were no biological or statistical differences between the control and treated groups with respect to clinical observation, feed consumption, water consumption, ophthalmologic examinations, body weights, and body weight gains. There were no treatment-related changes in hematology and clinical chemistry at the end of week 13. There were no differences in the functional observation battery, grip strength and locomotor activity assays between treated and control animals.

At necropsy, there were no treatment related findings on organ weights, macroscopic findings and histopathologic examinations. All microscopic findings were considered to be within the background incidence of findings reported in this age and strain of laboratory animals.

Appendix 7 – Certificate of Analysis of Tox Lot (UFC concentrate)

CERTIFICATE OF ANALYSIS

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

Name of Test Article: Brew 2 X3 Xylanase

Production/Strain Name: *Trichoderma reesei* MVS X3 #21

Production Site:

Genencor International Culture Collection Number: GICC 20005257

Designation of Lot Tested: 20128109

Description: Fermentation liquid – clear brown

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

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

RESULTS:

1. Activity: 268873 XBU/g
   (All activity analysis completed by BAMQC per the following: X3 Xylanase: R-SOP-AL-208 [X3 Xylanase Konelab Activity Assay]

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

   Total Proteins: 182.29 mg/ml
   Active Proteins: 136.20 mg/g
   TCA Proteins: 133.42 mg/ml
   % Total Organic Solids: 15.25 %
   (100% – moisture% – ash%) (100% - 84.59 % - 0.16 %)
   (Analysis completed by [redacted])

3. Specific gravity: 1.05 g/ml

4. pH: 4.98

5. Microbial analysis: Microbial analysis conducted b[redacted]r by outside laboratories.

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

6. Mycotoxin analysis: Completed by[redacted].

Total aflatoxin	< 5 ppb
T-2 toxin	< 25 ppb
Zearalenone toxin	< 50 ppb
Ochratoxin	< 10 ppb
Sterigmatocystin	< 40 ppm
Fumonisin	< 50 ppb

7. Heavy metals analysis (analysis completed by [redacted] using Inductively Coupled Plasma – Optical Emission Spectrometry or Direct Mercury Analysis)

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

8. Stability Data
The activity stability was analyzed in[redacted] under different conditions
Results are expressed as XBU/g

--Room Temperature (25°C)

Sample	T = 0	T = 5 hours	% of T = 0
20128109 (straight)	259304	267370	103.1
20128109 (diluted ½ in water)	133584	135107	101.1
20128109 (diluted ¼ in water)	68093	68582	100.7

--Refrigerated (4°C)

T = 0	T = 4 days	% of T = 0	T = 7 days	% of T = 0
262748	259377	98.7	259304	98.7

--Freezer (-20°C)

T = 0	T = 30 days	% of T = 0	T = 60 days	% of T = 0	T = 90 days	% of T = 0
262748	249765	95.1	254629	96.9	253678	96.5

(b) (6)

..... Date: 2/22/13

Christine Rechichi
BioAnalytical Group
Dupont Industrial Biosciences Division

(b) (6)

..... Date: 2/25/2013

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

Appendix 8 – GRAS Concurrence Letter from Dr. Michael W. Pariza

Michael W. Pariza Consulting LLC
7102 Valhalla Trail
Madison, WI 53719
(608) 271-5169
mwpariza@gmail.com

Michael W. Pariza, Member

June 13, 2014

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

RE: GRAS opinion on the intended use of DuPont's Xylanase X3 from *Aspergillus niger (tubingensis)* that is expressed in a non-pathogenic, non-toxigenic strain of *Trichoderma reesei*

Dear Dr. Sewalt,

I have reviewed the information you provided on DuPont's xylanase enzyme preparation, designated X3, which is expressed by *Trichoderma reesei* Brew2#21 (GICC 20005257), a production strain that has been genetically modified to over-express the native xylanase enzyme from *Aspergillus niger* 10M-61. The intended use of X3 are as a processing aid in brewing and the manufacture of potable alcohol, cereal beverages, and bakery, where the enzyme is either not present in the final food, or present at trace levels as inactive protein having no function or technical effect.

In evaluating X3, I considered the biology of *T. reesei* and *A. niger* and their history of safe use in food-grade enzyme manufacture; safety evaluation studies on the native X3 *A. niger* 10M-61 xylanase that was cloned into *T. reesei* Brew2#21 (GICC 20005257); safety evaluation studies on other food grade enzymes expressed by DuPont's safe lineage of *T. reesei* production strains, in particular the AkAA α-amylase produced by *T. reesei* LOH4AkAA paA (GICC 03387) which shares its immediate parent with *T. reesei* Brew2#21 (GICC 20005257); and information available in the peer-reviewed scientific literature; and the information that you provided regarding the safe lineage of the production organism, cloning methodology, manufacturing materials and procedures, and product specifications.

By way of background, *T. reesei* is used widely by enzyme manufacturers worldwide for the production of enzyme preparations that are, in turn, used in human food, animal feed, and

numerous industrial enzyme applications. DuPont's lineage of safe *T. reesei* production strains, including *T. reesei* Brew2#21 (GICC 20005257), was derived through a series of modifications from *T. reesei* QM6a, the original non-pathogenic and non-toxigenic wild-type parental strain used to produce this safe lineage of *T. reesei* enzyme production strains. Published literature, government laws and regulations, for example FR 64:28658-28362 (1999), reviews by expert panels such as FAO/WHO JECFA (1992), and DuPont's (legacy Genencor and Danisco) unpublished safety studies, all support the conclusion that the lineage to which these production strains belong is safe and suitable for use in the manufacture of food-grade and feed-grade enzymes.

Strains within this safe lineage are used to manufacture many food and feed enzymes, including chymosin, xylanase, cellulases, glucoamylase, α-amylase, β-glucosidase/cellulase, acid fungal protease, transglucosidase, lipase, and phytase. The enzyme products from 14 production strains within this safe lineage, and in two cases the production strains themselves, have been subjected to toxicology testing and rigorous safety evaluation in accordance with the Pariza-Johnson decision tree (Regulatory Toxicol. Pharmacol. 33: 173-186,2001). Some of these enzymes are also the subject of GRAS notification documents that are listed on the FDA GRAS Notice Inventory, for example GRN 00230, 00315, 00333 and 00372, all of which carry the decision statement, "FDA has no questions."

Aspergillus niger is also widely used by enzyme manufacturers worldwide for the production of enzyme preparations for use in human food, animal feed, and numerous other industrial enzyme applications. The X3 (xylanase) enzyme gene that was cloned into the *T. reesei* Brew2#21 (GICC 20005257) production strain was obtained from *A. niger (tubingensis)*10M-61, a non-pathogenic and non-toxigenic strain that has been used for decades to produce food-grade and feed-grade xylanase. Accordingly the native X3 enzyme has a history of safe use as a processing aid in food and feed applications.

The native X3 xylanase enzyme from *A. niger (tubingensis)*10M-61 was evaluated for acute, genotoxic and subchronic toxicity. No dose-related adverse events were observed in any of the studies. The NOAEL for native X3, calculated from the highest dose tested in a 90 day feeding study in Sprague-Dawley rats, was 1000 mg/kg bw/day, equivalent to 35 mg total protein/kg bw/day. The safety of X3 xylanase produced by *T. reesei* Brew2#21 (GICC20005257) was also evaluated for acute, genotoxic and subchronic toxicity. No dose-related adverse events were observed in any of the studies. Daily administration of the X3 xylanase produced by *T. reesei* Brew2#21 (GICC20005257) by gavage to female CD rats for 13 continuous weeks in rats did not result in overt signs of systemic toxicity. A NOAEL was established at 1000 mg TOS/kg bw/day.

Consumer exposure to the X3 xylanase expressed by *T. reesei* Brew2#21 (GICC 20005257) from all intended uses is estimated to be 2.428 mg TOS/kg bw/day. This exposure level is orders of magnitude below the NOAELs for the native X3 expressed by *A. niger (tubingensis)*10M-61, and the AkAA α-amylase that is produced by *T. reesei* LOH4AkAA paA (GICC 03387) which shares its immediate parent with *T. reesei* Brew2#21 (GICC 20005257). Thus, the required elements for evaluating the safety of the X3 xylanase expressed by *T. reesei* Brew2#21 (GICC 20005257) are met (MW Pariza and EA Johnson, Regulatory Toxicol. Pharmacol. 33: 173-186,2001).

The cloning techniques and methodologies employed to construct *T. reesei* Brew2#21 (GICC

20005257) are appropriate for use in the genetic modification of production strains for food ingredient manufacture. In addition, the manufacturing process including the ingredients used for fermentation, extraction and concentration of X3, and the specifications for X3, are appropriate for a food ingredient.

Based on the foregoing, I concur with the evaluation made by DuPont that the *T. reesei* Brew2#21 (GICC 20005257) production strain is safe and appropriate to use for the manufacture of food-grade X3. I further conclude that the X3 xylanase enzyme, manufactured in a manner that is consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food-grade specifications, is GRAS (Generally Recognized As Safe) for use as a processing aid in brewing and the manufacture of potable alcohol, cereal beverages, and bakery, where the enzyme is either not present in the final food or present at trace levels as inactive protein having no function or technical effect.

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

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

Sincerely,

(~~b)~~ (6)

Michael W. Pariza
Member, Michael W. Pariza Consulting, LLC
Professor Emeritus, Food Science
Director Emeritus, Food Research Institute
University of Wisconsin-Madison

SUBMISSION END