GR

ORIGINAL SUBMISSION

000001

GENENCOR
A Danisco Division

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

January 27, 2011

Dr. Robert Martin
Deputy Division Director
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

RECEIVED
JAN 31 2011
Division of
Biotechnology and
GRAS Notice Review

RE: GRAS Notification - Exemption Claim

Dear Dr. Martin,

Pursuant to the proposed 21C.F.R. § 170.36 (c) (l) Genencor, A Danisco Division, hereby claims that Glucoamylase enzyme preparation produced by *Trichoderma reesei* expressing the gene encoding glucoamylase from *Trichoderma reesei* 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

Genencor, A Danisco Division
925 Page Mill Road
Palo Alto, CA 94304

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

Glucoamylase (GA) enzyme preparation from *Trichoderma reesei* expressing the gene encoding the GA from *T. reesei* (TrGA).

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

Glucoamylase is used as a processing aid in grain processing, including the manufacture of corn sweeteners such as high fructose corn syrup (HFCS), brewing, potable alcohol manufacture, and lactic acid production.

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

This GRAS determination is based upon scientific procedures.

Proposed § 170.36 (c)(1)(v) Availability of information

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

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

Sincerely,

(b) (6)

Vincent Sewalt, PhD
Director of Regulatory Affairs
Genencor, a Danisco Division
650-846-5861 / vincent.sewalt@danisco.com

Enclosures (3 binders)

RECEIVED
JAN 31 2011
Division of
Biotechnology and
GRAS Notice Review

A Glucoamylase Enzyme Preparation Derived from *Trichoderma reesei* Expressing the Glucoamylase Gene from *Trichoderma reesei* Is Generally Recognized As Safe For Use in Food Processing

Notification Submitted by Genencor, a Danisco Division

January 27, 2011

GENENCOR

TABLE OF CONTENTS

1. GENERAL INTRODUCTION 3
1.1 EXEMPTION FROM PRE-MARKET APPROVAL 4
1.2 NAME AND ADDRESS OF NOTIFIER 4
1.3 COMMON OR USUAL NAME OF SUBSTANCE 4
1.4 APPLICABLE CONDITIONS OF USE 4
1.5 BASIS FOR GRAS DETERMINATION 4
1.6 AVAILABILITY OF INFORMATION FOR FDA REVIEW 4
2. PRODUCTION MICROORGANISM 5
2.1 PRODUCTION STRAIN 5
2.2 HOST MICROORGANISM 5
2.3 GLUCOAMYLASE EXPRESSION VECTOR 5
2.4 STABILITY OF THE INTRODUCED GENETIC SEQUENCES 6
2.5 ANTIBIOTIC RESISTANCE GENE 6
2.6 ABSENCE OF PRODUCTION MICROORGANISM IN PRODUCT 6
3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE 6
3.1 ENZYME IDENTITY 6
3.2 AMINO ACID SEQUENCE 6
4. MANUFACTURING PROCESS 7
4.1 RAW MATERIALS 7
4.2 FERMENTATION PROCESS 7
4.3 RECOVERY PROCESS 8
4.3.1 Concentration process 8
4.3.2 Formulation and standardization process 8
4.3.3 Quality control of finished product 8
5. COMPOSITION AND SPECIFICATIONS 8
5.1 QUANTITATIVE COMPOSITION 8
5.2 SPECIFICATIONS 9
6. APPLICATION 9
6.1 MODE OF ACTION 9
6.2 USE AND USE LEVELS 9
6.3 ENZYME RESIDUES IN THE FINAL FOODS 10
7. SAFETY EVALUATION 12
7.1 SAFETY OF THE PRODUCTION STRAIN 12
7.1.1 Safety of the host 12
7.1.2 Safety of the donor source 14
7.2 SAFETY OF THE MANUFACTURING PROCESS 14
7.3 SAFETY OF GLUCOAMYLASE 14
7.3.1 Allergenicity 14
7. 4. OVERALL SAFETY ASSESSMENT AND HUMAN EXPOSURE 23
7.4.1 Identification of the NOAEL 23
7.4.2 Human Exposure Assessment 23
7.4.3 Conclusion 26
8. BASIS FOR GENERAL RECOGNITION OF SAFETY 27
9. LIST OF REFERENCES 28
10. LIST OF APPENDICES 32

GENENCOR

1. GENERAL INTRODUCTION

The glucoamylase (GA) enzyme preparation under consideration is produced by submerged fermentation of *Trichoderma reesei* carrying the native *gla1* gene encoding the wild-type GA enzyme.

The enzyme product is intended for use in grain processing including the manufacture of corn sweeteners such as high fructose corn syrup, brewing, potable alcohol manufacture, and lactic acid production. In these applications, the Trichoderma GA (hereafter abbreviated as TrGA) will primarily be replacing GA from one of the other available commercial sources. In all of these applications, TrGA 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 glucoamylases currently in use include GAs derived from other microorganisms, most notably *Aspergillus oryzae*, *A. niger* and *Rhizopus sp.* The GA from *T. reesei* has been in commerce as a minor component of other *T. reesei* derived enzyme preparations, as GAs are endogenous to *T. reesei*. Only relatively recently however, enzyme preparations with commercially viable levels of TrGA production were introduced. Genencor first determined TrGA to be GRAS in 2006.

The accepted name of the principle enzyme activity is glucan 1,4-α-glucosidase. Other names used are amyloglucosidase; γ-amylase; lysosomal α-glucosidase; acid maltase; exo-1,4-α-glucosidase; glucose amylase; γ-1,4-glucan glucohydrolase; acid maltase; and 1,4-α-D-glucan.

The enzyme catalyzes hydrolysis of terminal (1→4)-linked α-D-glucose residues successively from non-reducing ends of the chains with release of β-D-glucose.

The EC number of the enzyme is 3.2.1.3, and the CAS number is 9032-08-0.

The information provided in the following sections is the basis of our determination of GRAS status of this TrGA 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.[1,2] The safety of the production organism (*T. reesei*) for this GA, 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.[3,4,5,6,7,8] The genetic modifications used to construct this production organism are well defined and are described in Section 2. The safety evaluation described in Section 7 shows no evidence to indicate that any of the cloned DNA sequences and incorporated DNA code for or express a harmful toxic substance.

GENENCOR

1.1 Exemption from Pre-market Approval

Pursuant to the regulatory and scientific procedures established in proposed 21 C.F.R. 170.36, Genencor, a Danisco Division, ("Danisco") has determined that its glucoamylases (GA) enzyme preparation produced by *Trichoderma reesei* expressing the gene encoding GA from *T. reesei* is a Generally Recognized as Safe ("GRAS") substance for the intended food application and is, therefore, exempt from the requirement for premarket approval.

1.2 Name and Address of Notifier

Genencor, A Danisco Division
925 Page Mill Road
Palo Alto, CA 94304

1.3 Common or Usual Name of Substance

Glucoamylase (GA) enzyme preparation from *Trichoderma reesei* expressing the gene encoding the GA from *T. reesei* (TrGA).

1.4 Applicable Conditions of Use

Glucoamylase is used as a processing aid in grain processing, including the manufacture of corn sweeteners such as high fructose corn syrup (HFCS), brewing, potable alcohol manufacture, and lactic acid production.

1.5 Basis for GRAS Determination

This GRAS determination is based upon scientific procedures.

1.6 Availability of Information for FDA Review

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

GENENCOR

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 *T. reesei* glucoamylase gene for the production and secretion of TrGA 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 Good Industrial Large Scale Practice (GILSP) worldwide. It also meets the criteria for a safe production microorganism as described by Pariza and Johnson[1]. Two expression cassettes were used to introduce the *T. reesei* gene encoding GA under the regulation of the native *T. reesei cbhI* (cellobiohydrolase 1) promoter and terminator. One of the expression cassettes used the *A. nidulans amdS* gene as a selectable marker and the other used the endogenous *T. reesei* acetolactate synthase (*als*) gene as a selectable marker. Both 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[9]. 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*.[10,11]

2.3 GA Expression Cassettes

Two GA expression cassettes were used. The first cassette contained the *T. reesei cbh1* promoter and terminator, genomic *glaI* DNA encoding the *T. reesei* GA, and the *Aspergillus nidulans amdS* gene, encoding acetamidase, as a selectable marker for transformation of *T. reesei*. The second cassette consisting of the *T. reesei cbh I* promoter and terminator, *gla1* genomic DNA, , and *T. reesei als* gene marker, encoding acetolactate synthase, was used for retransformation of one of the primary *T. reesei* transformants confirmed to express the first GA expression cassette, resulting in the final production strain 70H2-TrGA #32-9.

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 GA cassettes and the absence of bacterial vector DNA.

GENENCOR

2.4 Stability of the Introduced Genetic Sequences

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

2.5 Antibiotic Resistance Gene

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

2.6 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)[3] is satisfactorily addressed.

3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1 Enzyme Identity

IUBMB Nomenclature[12]:	Glucan 1,4-α-glucosidase
IUBMB Number:	3.2.1.3
CAS Number:	9032-08-0
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:	amyloglucosidase; γ-amylase; lysosomal α-glucosidase; acid maltase; exo-1,4-α-glucosidase; glucose amylase; γ-1,4-glucan glucohydrolase; acid maltase; and 1,4-α-D-glucan.

3.2 Amino Acid Sequence

The amino acid sequence of the *Trichoderma reesei* GA enzyme is known and is shown in Appendix 1A. The sequence of TrGA is similar to various other GAs isolated from commercially relevant fungi, e.g., it is 56% homologous with *Aspergillus niger* GA, the most predominant GA currently in commerce (see Appendix 1B). Overall, the listed GAs share from 47 to 68% homology with one another.

GENENCOR

4. MANUFACTURING PROCESS

This section describes the manufacturing process for the GA enzyme which follows standard industry practice[13,14,15]. For a diagram of the manufacturing process, see Appendix 2. The quality management system used in the manufacturing process complies with the requirements of ISO 9001. The enzyme preparation is also manufactured in accordance with FDA's current Good Manufacturing Practices ("cGMP") as set forth in 21 C.F.R. Part 110.

4.1 Raw Materials

The raw materials used in the fermentation and recovery process for this GA concentrate are standard ingredients used in the enzyme industry[13,14,15]. All the raw materials conform to the specifications of the Food Chemicals Codex, 7th edition, 2010 ("FCC")[16], 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. Danisco 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 submission to FDA on antifoams and flocculants dated April 24, 1998. The maximum use level of the antifoam in the production process is $\leq$0.15%.

4.2 Fermentation Process

The GA 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.

GENENCOR

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 and consists of both concentration and formulation processes.

4.3.1 Concentration 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. Formulation; and
4. Polish filtration.

4.3.2 Formulation and standardization process

The ultrafiltered concentrate is stabilized by final formulation to contain ~10% glucose, ~0.3 % sodium benzoate, ~0.11% potassium sorbate, at pH 4.0 – 4.5. The remaining is water.

4.3.3 Quality control of finished product

The final GA 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)[17] in 2006 and the Food Chemical Codex 7th edition (FCC)[16]. 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:	Avg. ~ 570 GAU/g
Enzyme protein:	Avg. ~15%
Total Organic Solids:	Avg. ~16%
	(1 mg enz. protein = 1.04mg TOS)
Potassium sorbate:	~0.11%
Glucose:	~0-10%
Sodium benzoate:	~0.3 %
pH:	4.0 – 4.5

5.2 Specifications

TrGA meets the purity specifications for enzyme preparations set forth in the FCC 7th edition[16]. 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[17], including the absence of T2-toxin, the only mycotoxin of concern for this species.

6. APPLICATION

6.1 Mode of Action

As noted above, GA catalyzes hydrolysis of terminal 1,4-linked α-D-glucose residues successively from non-reducing ends of the chains with release of β-D-glucose.
It can also hydrolyze 1,6-α-D-glucosidic bonds when the next bond in the sequence is 1,4.

6.2 Use and Use Levels

6.2.1 Uses

Grain Processing:

GA is used to saccharify liquefied corn starch for the manufacture of corn sweeteners such as high fructose corn syrups (HFCS).

Brewing and Potable Alcohol:

GA is used to maximize the conversion of starchy substrate to fermentable carbohydrate. It will be used in the extraction and saccharification of starch (mashing) from malted cereal, cereal and other plant sources (includes barley, corn, wheat, rye, milo, rice, tapioca and potatoes). The resultant process liquors (worts) are fermented, typically by a yeast, to produce ethanol (and

sometimes organic acids). TrGA may also be used in the fermentation vessel hydrolyzing liquefied starch to glucose which is fermented to ethanol.

Lactic acid Production:

Trichoderma GA will be used in lactic acid manufacture processes similar to those used in potable alcohol manufacture. The lactic acid is used in a wide range of food applications such as bakery products, meat, poultry and fish products, salad and dressings, and dairy.

6.2.2 Use Levels

Grain Processing:

In a typical saccharification, the recommended dosage for TrGA is 0.2 – 0.5 kg of product per metric ton of dry starch substance.

Brewing and Potable Alcohol:

In a typical mash brewing application, the recommended dosage for TGA is 2.5 - 5.0 kg of product per metric ton of grist (e.g. malted barley). Approximately 17 kg of grist (e.g. malted barley) are used to make 1 hectoliter (100 liters) of finished beer. In potable alcohol manufacture, the use rate is lower and the recommended dosage for TrGA is 0.3 - 0.5 kg of product per metric ton of equivalent dry starch substance (or approximately 0.6 - 1.0 kg of product per metric ton of equivalent grist).

Lactic acid Production:

In a typical lactic acid production, the recommended dosage for TrGA is 0.5 kg to 3 kg of product per metric ton of dry substrate. The use levels of lactic acid into various food applications are listed in section 7.4.2.

6.3 Enzyme Residues in the Final Foods

Genencor expects TrGA to be inactivated or removed during the subsequent production processes for all applications.

In grain processing to produce corn sweeteners such as HFCS, the enzyme is added in the saccharification step, to liquefied starch. After the conversion to high glucose syrup, it is then filtered to remove the insoluble solids. The clear high-glucose solution is treated with carbon between 75 to 85 C° followed by treating with ion-exchange resins to remove salts[18, 19, 20]. The syrup is then evaporated to increase solids. In HFCS manufacture, evaporation is to 50 % dry solids, which is followed by reaction with an immobilized glucose isomerase enzyme for producing fructose. The high fructose syrup is again treated with carbon and ion-exchange resins. The various additional purification steps (filtration, carbon treatment, ion exchange) to which corn syrups are subjected effectively remove all protein. The minimum enzyme protein

carry-over into the syrup combined with the low enzyme product use level in corn syrup manufacture results in negligible TrGA protein in corn syrups such as HFCS.

In brewing, TrGA is added in mashing, before the boil (and before final beer pasteurization). Because of denaturation and aggregation of proteins during the mash and subsequent wort boiling steps, much if not all of the TrGA enzyme is expected to be removed in post boiling clarification processes. When the enzyme is added into the fermentation vessel the beer passes through a pasteurization step targeted in the range of 10-11 PU's (1 PU equals 140 °F for 1 min), typically at 60 °C for 10 min, but variations in temperature and heating time combinations can accomplish pasteurization as well. As such, beer pasteurization is not guaranteed to inactivate TrGA, as this is also product matrix dependent including pH and alcohol content. However, as residual GA activity would potentially adversely impact beer quality and consistency by acting on residual carbohydrate substrate, it is generally recommended and common practice to assay for residual GA activity to ensure inactivation. Given the mild pasteurization conditions the enzyme protein likely remains soluble and, therefore, the inactive enzyme protein is not removed in final beer filtration.

In potable alcohol applications, TrGA is typically added in the saccharification or the fermentation step. A separation and distillation steps are used subsequent to these steps. Upon conclusion of the fermentation, solids are separated from the fermentation slurry and any TrGA 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 TrGA 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 TrGA enzyme is denatured and any residuals are effectively removed from the alcohol such that no protein, including TrGA, remains in the final product.

In lactic acid production, TrGA is added during the saccharification step or alternatively, during the fermentation phase of the lactic acid production. The lactic acid then goes through several separation/purification steps post fermentation. First, at the end of the fermentation, calcium carbonate is added to the fermentor to neutralize the acid produced. The broth is then heated to above 82°C to inactivate the lactic acid fermentation organisms. As the Tm of GA is less than 74°C, the heating step effectively inactivates the enzyme by denaturation.

Subsequent lactic acid purification and separation steps include:

1) acidulation to convert calcium lactate back to lactic acid;
2) demineralization (ion exchange) to remove the impurities with electrical charge: cations (calcium), anions (sulfate), and light organic acids (formic acid, acetic acid);
3) concentration (vacuum evaporation) to remove the water;

4) direct distillation to remove the impurities with higher boiling point as these remain in the residue; distillation will also result in removal of any residual TrGA protein.
5) carbon treatment to remove the impurities which have an affinity to carbon (e.g. color & odor).

7. SAFETY EVALUATION

7.1 Safety of the Production Strain

The safety of the production organism must be the prime consideration in assessing the safety of an enzyme preparation intended for use in food[1]. 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[4]. Pariza and Foster[2] 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[21] that *T. reesei* be placed into synonymy with *T. longibrachiatum*. Later however, evidence emerged indicating that the two species are not identical[22]. The proposal by Kuhls *et al.*[10] 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 documents, but they refer to essentially the same microorganism species.

A review of the literature search on the organism (1972 - 2009) 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 reported the isolation from *T. reesei* strain QM 9414 a peptaibol compound that exhibited antibiotic activity[23]. Their work was confirmed by another group that found

evidence of peptaibol production in two other *T. reesei* strains[24]. 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. 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[17].

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.[25] and Blumenthal[26]. 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 paper[1] and Olempska-Beer *et al.*[27]. 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), France, Australia/New Zealand, China, and Japan.

The production organism of the GA enzyme preparation, the subject of this submission, is *T. reesei* strain 70H2-TrGA #32-9. The strain has been genetically modified by inactivation of several cellulase genes, NTG mutagenesis and selection to lower its viscosity during fermentation, and by overexpression of the native *T. reesei* GA gene for the production and secretion of *T. reesei* GA 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. Genencor 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 GA produced by this specific production strain, 70H2-TrGA #32-9, has been determined to be non-toxicogenic through the following toxicology tests: 1) acute dermal irritation study in rabbits, 2) acute eye irritation/corrosion study in rabbits 3) acute oral toxicity in rats 4) bacterial reverse mutation – Ames assay, 5) *In vitro* mammalian chromosomal aberration test performed with human lymphocytes, and 6) 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 three of the products from this strain lineage: chymosin, transglucosidase and acid fungal protease, and FDA has issued "no questions" letters in response to each (see GRAS Notice 230 [28],315[29], 333[30] respectively).

From the information reviewed, it is concluded that the production organism *T. reesei* strain 70H2-TrGA #32-9 provides no specific risks to human health and is safe to use as the production organism of TrGA. The strain is non-pathogenic and non-toxigenic.

7.1.2 Safety of the donor source

In this case, the donor organism is the parent strain of the host strain, *T. reesei* QM6a, therefore the safety assessment is the same as for the host.

7.2 Safety of the Manufacturing Process

The manufacturing process for the production of TrGA 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 GA 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 Food Chemicals Codex , 7th Edition[16] and the general specifications for enzyme preparations used in food processing proposed by JECFA[17].

7.3 Safety of *Trichoderma reesei* Glucoamylase

7.3.1 Allergenicity

According to Pariza and Foster[2], 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 enzyme residues in foods do not represent any unacceptable risk to consumers. Further, in a 2006 Danish study 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[31]." A wide variety of enzyme classes and origins was tested in that study including glucoamylase. Although the GA included in the publication was produced from *Aspergillus niger,* as discussed in section 3.2 and shown in Appendix 1B, the two glucoamylases share great sequence homology.

Despite this lack of general concern, the potential that TrGA could be a food allergen was assessed by comparison with sequences of known allergens and is summarized here. The *T. reesei* glucoamylase (GA) mature peptide sequence was subjected to FASTA alignment of the sequence with known allergens using the SDAP database (http://fermi.utmb.edu/SDAP/sdap_who.html)[32] containing 887 allergens (listed in http://fermi.utmb.edu/cgi-bin/SDAP/sdap_01). The search did not reveal any homology with listed allergens.

Guidelines developed by FAO/WHO (2001)[33] as discussed and modified by the Codex Alimentarius Commission [34] and republished by FAO/WHO (2009)[35] 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 [36] but that the 2001 Joint FAO/WHO Expert Consultation[33] suggested moving from 8 to 6 identical amino acid segments in contiguous searches. Following the FAO[33] guidelines but taking the cautionary Codex [34,35] comments into account, two additional searches against the SDAP database were performed.

A search for 80-amino acid stretches with greater than 35% identity to known allergens revealed two matches for the *T. reesei* GA; Pen ch 13 (fungi, Ascomycota Eurotiales; *Penicillium citrinum,* alkaline serine protease) and Lol p 5(Poales; *Lolium perenne,* rye grass). Both allergens are listed as non-food allergens by the IUIS [37](International Union of Immunological Societies). Although cross-reactivity of inhalation or dermal allergens with food allergens cannot be completely excluded, and a few food enzymes have indeed demonstrated cross-reactivity to inhalation or dermal allergens via skin prick testing, none of those tested to date have been demonstrated to be active via the oral route.

A secondary search for exact matches of short stretches (≥ 8 amino acids) of sequences that could serve as potential IgE binding sites resulted in zero matches.

Furthermore, the carry-over of TrGA protein into the final food from the use of the enzyme in the manufacture of corn syrups (HFCS), beer, potable alcohol, and refined lactic acid is negligible as most of these processes include various purification steps that remove residual protein (except in case of brewing where inactive enzyme may remain in the beer).

To further support that the use of *T. reesei* GA to replace existing GAs in commerce will not alter the allergenicity risk profile of glucoamylases used even in those applications where the enzyme protein remains present in the final food, brief sequence-homology based allergenicity assessments were conducted for three main glucoamylases used in commerce: *Aspergillus niger* GA, *Rhizopus oryzae* GA, and *Aspergillus oryzae* GA. The AA sequences of these three GAs were subjected to the same screening searches with >35% identity over 80 AA stretches. The GenInfo Identifiers (GI) for the respective glucoamylases in the GenBank database and the sequence match results are summarized below.

GENENCOR

Amino Acid Sequence homology of various glucoamylases to known allergens

Source organism	Allergen matches with >35% identity over 80AAs stretches
>gi|49615615|gb|AAT67041.1| glucoamylase [*Aspergillus niger*]	Asp f 16 (*A. fumigatus*) —respiratory allergen
>gi|218035:26-604 glucoamylase [*Rhizopus oryzae*]	Asp f 16 (*A. fumigatus*) —respiratory allergen
>gi|217825|dbj|BAA00841.1| glucoamylase [*Aspergillus oryzae*]	Hor v 5.0101 - barley pollen allergen

Similar to the respiratory allergen matches found with the *T. reesei* GA, all three GA AA sequences shown above result in matches with respiratory allergens. However, oral allergenicity concerns have not been associated with these GAs, and all three enzymes have GRAS status per GRAS Notices (GRN 89 for *A. niger* GA[38], and GRN 90 for *A. oryzae* GA[39]) and GRAS affirmation (21CFR173.130 for *R. oryzae* GA)[40]. Moreover, the reported lack of oral allergenicity of *A. niger* GA in human testing with sensitive individuals[31] discussed above reduces the likelihood that other GAs would be active via the oral route. Overall, replacing these GAs in the described applications with *T. reesei* GA will not result in any increase in existing human exposure to glucoamylase enzyme preparations and will not elevate the risk of food allergenicity.

In conclusion, using a weight-of-evidence approach, *T. reesei* GA is not likely to pose a risk of food allergenicity as:

1) no hits with known food allergens were found using the 35% match over 80-amino acids criterion;
2) no matches with short contiguous sequence stretches of known allergens were found.;
3) the amount of enzyme carried over from grain processing, brewing, potable alcohol and lactic acid production into the final food products is negligible as described in section 6.4; and
4) several other commercial GAs that are either GRAS affirmed or GRAS notified have the same allergenicity profile as *T. reesei* GA, and these GAs are not associated with oral allergenicity or (for *A niger* GA) specifically disproven not to be orally active; as such, TrGA does not exhibit a higher allergenicity risk than any of these other GAs in commerce.

Danisco's evaluation as well as published studies support the conclusion that TrGA is extremely unlikely to be a food allergen. As for all enzyme products, the MSDS for the GA product includes a precautionary statement that inhalation of enzyme mist/dust may cause allergic respiratory reactions, including asthma, in susceptible individuals on repeated exposure.

7.3.2 Safety of use in food

In addition to the allergenicity assessment described above, the safety of this GA has also been established using the Pariza and Johnson (2001) decision tree[1], see Appendix 5. Although the Pariza and Johnson evaluation resulted in the conclusion to accept the enzyme preparation as

safe without toxicology testing, the safety of the enzyme preparation was further confirmed through unpublished toxicological testing as described below. The toxicology testing was conducted to be able to use the results in countries where toxicology testing is required for enzyme preparation approval.

7.3.3 Safety 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.

Danisco Genencor 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. Danisco Genencor has also conducted many safety studies on *T. reesei* derived enzymes (see Appendix 3).

In addition, Genencor has conducted six studies on the *T. reesei* GA enzyme produced from *T. reesei* 70H2-TrGA #32-9, the subject of this safety assessment. The test material contained 199.63 mg enzyme protein/ml and 20.73% Total Organic Solids (TOS) (1 mg enzyme protein = 1.038 mg TOS). Summaries are included below.

A. Acute dermal irritation study in rabbits (sequential approach)

Procedure
The objective of this study was to assess the local irritant effect of TrGA. This study was conducted according to the method recommended in the OECD Guideline No. 404, April 2002 and evaluated according to Commission Directive 2001/59/EC of 6 August 2001.

In the initial test, the back of one rabbit was divided into 4 test sites. Three sites were used for test material application whereas the fourth test site served as control (vehicle only). All test sites were observed at 3 minutes and at 1 and 3 hours post application. A confirmatory test was conducted later with two rabbits and reading was made at 1, 24, 48 and 72 hours post application.

Results
No deaths or overt signs of toxicity were observed in this study. No effects on feed consumption and weight gain were recorded. No erythema, eschar or edema was observed at these test sites at any of the examinations throughout the study. The primary irritation score (PIS) for erythema was 0.0 and the PIS for edema was also 0.0

Evaluation
On the basis of the results obtained in this study, TrGA is classified as non-irritant according to OECD Guideline No 404, "Acute Dermal Irritation/Corrosion", April 2002, and the Official

Journal of the European Communities, L 110 A, volume 36, 4 May 1993 § 3.2.5 ("C", R35 or R34) and § 3.2.6.1 ("Xi", R38).

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

Procedure
The objective of this study was to assess the ocular irritation potential of TrGA. 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.

In the initial test, the test material was applied at 0.1 ml to the left eye and the grade of ocular reaction was recorded at 1, 24, 48 and 72 hours later. The right eye served as control. 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. A confirmatory test was conducted with 2 rabbits. After termination of the study, 72 hours after treatment, the animals were killed.

Results
In the confirmatory assay with 2 rabbits, discharge was present (score 1) at the 1-hour period but disappeared at subsequent observations (24, 48 and 72 hours after treatment). The primary irritation score was 0.0

Evaluation
The primary eye irritation score was 0.0. According to the EEC Directive published in: EEC Directive published in: "Official Journal of the European Communities" No: L 383 A, volume 35, 29.12.1992, part B5: Acute toxicity (eye irritation) and No: L 110 A, volume 36, 04.05.1993, part 3.2.6.2 Ocular lesions (which is implemented in Commission Directive 2001/59/EC of 6 August 2001), TrGA is not classified as eye irritating.

C. **Acute oral toxicity in rats – Fixed dose procedure**

Procedure

The objective of this study was to assess the acute toxicity of TrGA when administered as a single oral dose followed by a 14-day period of observation. The information is used for both hazard assessment and ranking purposes. The study was initiated with a sighting study using 10 ml/kg (1996.3 mg total protein/kg bw). The main study was performed in four additional female rats given a single dose of 10 ml/kg bw (corresponding to 2072 mg TOS/kg bw).

This study was conducted according to OECD Guideline No. 420 (Acute oral toxicity – Fixed dose procedure), December 2001 and in compliance with the OECD Principles of Good Laboratory Practice (as revised in 1977).

Results

No mortality was recorded in this study. There were no overt signs of systemic toxicity throughout the 14-day observation period and at necropsy.

Evaluation

Under the conditions of this study, the oral LD_{50} was > 1996.3 mg protein/kg bw (corresponding to 2072 mg TOS/kg bw) which was the highest possible dose at dose volume 10 ml/kg, using the undiluted test item. Based on a LD_{50} of 1996.3 mg protein/kg bw, TrGA is classified to GSH category 5 according to Annex 2 and Annex 3 in the OECD Guideline.

D. Bacterial Reverse Mutation Assay – Ames assay.

Procedure

The objective of this assay was to assess the potential of 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. The test material was tested both in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). The tests were performed using the "treat and plate" method to avoid the possibility of interference from histidine in the test article. In the treat and plate method, various dose levels of TrGA (50, 160, 500, 1600 and 5000 µg/plate) were mixed with a concentrated bacterial suspension and nutrient broth. These mixtures were incubated at 37°C with gentle shaking for 3 hours. At the end of the 3-hour period, the bacteria were centrifuged, the supernatant was removed and the bacteria were resuspended in 2 ml buffer. The cultures were then centrifuged, the supernatant removed and the bacteria resuspended a second time in buffer and top agar was added. The contents of each tube were mixed and spread on selective agar plates. The plates were then incubated for 72 hours at 37°C and then scored for revertants and viability.

A preliminary toxicity test was performed in strain TA 98. Subsequently, two independent main tests were performed with all 5 strains in both presence and absence of S-9 mix. Triplicate plates were used at each test point. Five dose levels of TrGA were used in the main tests and ranged from 50 to 5000 µg/plate. All dose levels were expressed in terms of total protein. The highest dose level tested (5000 µg/plate) is the maximum required by the OECD guideline. The positive controls used for assays without S-9 mix were sodium azide, 2-nitrofluorene, 9-amino acridine and cumene hydroperoxide and the positive control used for assays with S-9 mix was 2-aminoanthracene. Negative control plates were treated by the addition of sterile saline solution.

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.

Results

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. 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. 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.

Evaluation

Under the conditions of this assay, TrGA has not shown any evidence of mutagenic activity in the Ames assay.

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

Procedure

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). TrGA concentrate 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 tests. Five concentrations of TrGA were used in the preliminary assay and at least 3 dose levels were then selected for the two main assays with the highest dose level clearly inducing a toxic effect (50% reduction in mitotic index). In the absence of cytotoxicity, the highest dose selected would be 5000 µg/ml, as recommended by the OECD guideline.

In the first main test, all cultures (with or without S-9 mix) were treated for 3 hours. In the second main test, cultures without S-9 mix were treated for 20 hours and those with S-9 mix for 3 hours.

All cultures (with and without S-9 mix) were harvested 20 hours (1.5 normal cell cycles) after the start of 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. At the harvest time, all cultures were centrifuged and the supernatant discarded. The cell pellets were resuspended in a KCl solution, incubated for 10 minutes, centrifuged and the supernatant removed. The cells were then fixed on slides, stained and scored for chromosomal aberrations:

a. Cytotoxicity was evaluated using the mitotic index (number of cells in mitosis/1000 cells examined. From these results, a dose level causing a decrease in mitotic index of 50% was selected as the highest dose in the main tests.

b. Metaphase analysis (i.e. evaluation of chromosomal aberration) was conducted on 100 metaphases per dose level.
c. Daunomycin C and cyclophosphamide were used as positive controls for cultures without S-9 mix and with S-9 mix, respectively.

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.

Results
TrGA did not cause marked cytotoxicity at any concentration tested in the preliminary study. Consequently, dose levels of 1250, 2500 and 5000 µg/ml were selected for the two main assays.

All concentrations are expressed in terms of the total protein content of the test item. The highest concentration tested (5000 µg/ml) was the maximum dose level recommended by the guideline. 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.

Evaluation
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.

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

Procedure
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. Groups of 10 rats/sex each were gavaged daily with 0 (0.9% saline), 10, 50 or 150 mg enzyme protein/kg body weight in a constant volume of 5 ml/kg body weight corresponding to 0, 10.4, 51.8 or 155.5 mg TOS/kg bw/day.

Animals of the same sex were pair-housed in transparent polycarbonate cages with softwood sawdust as bedding and had access to water (via bottle) and feed *ad libitum*. For environmental enrichment, the animals were provided a supply of Aspen Wood Wool at each change of bedding. All groups were housed under controlled temperature, humidity and lightning conditions.

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

in the control and high dose groups at study termination. Hematology was conducted on Day 0, 30 and 90. A functional observation battery consisting of detailed clinical observation, reactivity to handling and stimuli and motor activity examination was conducted during week 13 for the control and high dose rats. Clinical chemistry was evaluated at study termination prior to necropsy on all groups. After a thorough macroscopic examination, selected organs were removed, weighed and processed for future histopathology examination. Microscopic examination was initially conducted on selected organs from control and high dose animals. Due to questionable findings in males, the microscopic examination of the kidneys and testes was extended to the low and mid dose groups.

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.

Results

One control male (# 3) was sacrificed for animal welfare reasons on day 75 of the study due to the presence of a fast growing subcutaneous tumor, which impeded the animal's health. 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. 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 histopathology 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.

Evaluation and conclusion
Daily administration of TrGA by oral gavage for 90 consecutive days did not result in adverse systemic toxicity or adverse effects on clinical chemistry, hematology, functional observation tests and macroscopic and histopathology examinations. Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established at the highest dose tested, 150 mg enzyme protein/kg bw/day corresponding to 155.5 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 rats, a NOAEL was established at 150 mg total protein/kg bw/day (equivalent to 155.5 mg TOS/kg bw/day or 589.5 GAU/kg bw/day). The study was designed based on OECD guideline No. 408 and conducted in compliance with both the FDA Good Laboratory Practice Regulations and the OECD Good Laboratory Practice. Since human exposure to TrGA is through oral ingestion, selection of this NOAEL is thus appropriate.

NOAEL = 150 mg total protein/kg bw/day
= 155.5 mg TOS/kg bw/day
= 589.5 GAU/kg bw/day

7.4.2 Human Exposure to TrGA

Estimation of daily consumption of TrGA from its potential uses in major commodities is based on 2008 USDA data
(http://www.ers.usda.gov/data/foodconsumption/Spreadsheets/beverage.xls).

7.4.2.1 Brewing

TrGA is intended for brewing at the application rate of 2.5 to 5.0 kg of enzyme product per metric ton of grist (e.g., malted barley). In this assessment, an application rate of 5.0 kg of enzyme product is used to represent a worst case scenario. The enzyme product contains 570 GAU/g.

5.0 kg enzyme product per 1 m3 grist =
5.0 g enzyme product per 1 kg grist

Approximately 17 kg of grist are needed to make 100 liters of beer. Hence,
5.0 g enzyme product/kg grist X 17 kg =
85.0 g enzyme product per 100 liters of beer =

0.85 g enzyme product/liter of beer =
0.85 g enzyme product/liter of beer X 570 GAU/g enzyme product =
485 GAU/liter of beer =
0.485 GAU/ml of beer

Based on the USDA Continuing Survey of Food Intakes (2008), the per capita intake (apparent consumption) of beer is 21.7 gallons or 82 liters. The daily intake is calculated as 225 ml. Hence, the daily exposure to TrGA through consumption of beer and ale is:

0.485 GAU/ml X 225 ml = 109 GAU/day.

Using 65 kg body weight as default (male and female combined), then the maximum daily intake Is:

109 GAU/day: 65 kg = 1.68 GAU/kg bw/day.

The daily intake of 1.68 GAU/kg bw/day represents a worst case scenario assuming that:

a. TrGA is applied at the maximum rate of 5 kg/metric ton of grist (e.g. malted barley)
b. 100% of TrGA is transferred into beer
c. 100% of beer consumed is manufactured from grist treated with TrGA.

7.4.2.2. Potable Alcohol

TrGA is intended for the manufacture of potable alcohol manufacture. TrGA is used for the saccharification of liquefied starch to glucose. In these applications, TrGA will be used at 0.50 kg enzyme product per metric ton of equivalent starch dry. It is estimated that the ethanol yield from 1 metric ton of starch dry is 650 L potable alcohol. The enzyme product contains 570 GAU/g.

0.5 kg enzyme product per 1 metric ton of starch =
0.5 kg enzyme product per 1000 kg of starch =
0.5 kg enzyme product per 650 L of potable alcohol =
0.77 g enzyme product per L potable alcohol =
438 GAU per L potable alcohol =
(Since 1 g of enzyme product contains 570 GAU)
0.44 GAU per ml of potable alcohol

Based on the USDA, Agricultural Research Service (2008), the per capita intake of spirits is 1.4 gallons or 5.30 liters. The daily intake is calculated as 14.5 ml. The USDA Survey of Food Intake by Individuals (1988) cites 12.5 ml pure alcohol as the daily consumption (www.barc.usda.gov/bhn/foodsurvey). In this safety assessment, the higher value of 14.5 ml/day is used to represent a worst case scenario. Using 65 kg as default body weight, the daily consumption of pure alcohol is:

14.5 ml pure alcohol/day: 65 kg = 0.22 ml/kg bw/day.

To represent a worst case scenario, it is assumed that

a. 100% of potable alcohol (spirits) consumed daily are manufactured from dry starch treated with TrGA
b. 100% TrGA remains in the potable alcohol, although none is expected, hence:

Estimated maximum daily intake of TrGA from alcohol:
0.44 GAU/ml X 0.224 ml/kg bw/day =
0.10 GAU/kg bw/day.

7.4.2.3 Corn Sweeteners

TrGA is used in grain processing in the manufacture of corn sweeteners including high fructose corn syrup (HFCS), corn syrup, and dextrose. Total consumption of enzyme preparation residues may be estimated based on the assumption that all corn sweeteners in the US are produced using TrGA. Based on the information provided by the USDA in 2008 (www.ers.usda.gov/briefing), the estimated per capita consumption of corn based syrup and sweeteners is:

High fructose corn syrup =	47.1 g/day
Other sweeteners =	15.6 g/day
Total Daily Consumption =	62.7 g/day

Using 65 kg as the default for body weight for males and females, the human consumption of corn sweeteners is:

62.7 g/day : 65 kg = 0.96 g HFCS/kg bw/day

The application rate of TrGA is 174,000 GAU/metric ton =
174,000 GAU/1000 kg =
174 GAU/kg or 0.174 GAU/g.

Maximum concentration of TrGA in corn sweeteners = 0.174 GAU/g.

Estimated Maximum Daily Intake of TrGA from corn sweeteners
0.96 g corn syrup-sweeteners/kg bw/day X 0.174 GAU/g =
0.17 GAU/kg bw/day.

7.4.2.4. Lactic acid manufacture

Lactic acid is used as a preservative in the manufacture of many foods including preserved meat, cooked chicken, canned fish, yogurt, and bakery at different application rates (see attached spread sheet). TrGA is used in the manufacture of lactic acid at an application rate of 0.5 to 3 kg per metric ton. In this risk assessment, the highest application of 3 kg/m3 is used to represent a worst case scenario. The total consumption of TrGA from various applications of lactic acid is calculated as 0.098 mg/kg/day (see attached spread sheet). Since 1 g of TrGA corresponds to 570 GAU, the maximum daily consumption of TrGA from all applications of lactic acid is 0.056 GAU/kg bw/day.

GENENCOR

7.4.2.5. Cumulative Exposure from foods

Although it is expected that residues of a processing aid in the final products would be inactivated and negligible after processing, a worst-case-scenario approach is used in this risk assessment assuming that:

(1) 100% of the above commodities are treated with TrGA enzyme product
(2) Human's consumption consists of only commodities treated with TrGA
(3) 100% of TrGA is not removed during processing,

Maximum Daily Exposure to TrGA =
1.68 GAU/kg bw/day from brewing
0.10 GAU/kg bw/day from potable alcohol
0.17 GAU/kg bw/day from corn sweeteners
0.056 GAU/kg bw/day from lactic acid application

Total = 2.006 GAU/kg bw/day from all commodities
Maximum Daily Exposure = 0.56 mg TOS/kg bw/day

Based on the results from the 90-day oral (gavage) feeding study cited above, a margin of safety can be calculated based on the following equation:

$$\text{Margin of safety} = \frac{\text{No observed adverse effect level}}{\text{Maximum daily exposure}}$$

$$\text{Margin of safety} = \frac{155.5 \text{ mg TOS kg bw/day}}{0.56 \text{ mg TOS/kg bw/day}} = 277$$

7.4.3 Conclusion

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

Daily administration of TrGA by gavage for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at 150 mg total protein/kg bw/day (equivalent to 155.5 mg TOS/kg bw/day or 589.5 GAU/kg bw/day).

Even under the worst case scenario that TrGA is applied at the maximum rate and the enzyme is not destroyed and/or removed during processing, the use of TrGA in grain processing, brewing,

manufacture of potable alcohol and lactic acid manufacture is not expected to result in adverse effects to humans. Under the conditions of these safety/toxicology studies, a margin of safety of 277 exists between the systemic NOAEL of 155.5 mg TOS/kg bw/day (589.5 GAU/kg bw/day)and the estimated maximum daily human cumulative exposure level of 0.56 mg TOS/kg bw/day (2.27 GAU/kg bw/day)

8. BASIS FOR GENERAL RECOGNITION OF SAFETY

As noted in the Safety sections above, *Trichoderma reesei*, and enzyme preparations derived there from, including cellulase, 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 Genencor's 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[1] the TrGA enzyme preparation was determined to be acceptable for the proposed uses.

The safety studies conducted on the TrGA produced by the production strain described in this submission established a NOAEL at 150 mg total protein/kg bw/day (equivalent to 155.5 mg TOS/kg bw/day). Based on a worst-case scenario, the use of TrGA in grain processing including the manufacture of corn sweeteners such as high fructose corn syrup, brewing and manufacture of potable alcohol, and lactic acid production is not expected to result in adverse effects to humans. A safety margin of 277 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 Genencor, the company has concluded that *T. reesei* Glucoamylase from *Trichoderma reesei* strain 70H2-TrGA#32-9 is safe and suitable for use in grain processing including the manufacture of corn sweeteners such as high fructose corn syrup, brewing, potable alcohol manufacture, and lactic acid production. 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 6) for its intended uses.

GENENCOR

9. LIST OF REFERENCES

1. 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.

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

3. 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.

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

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

6. 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.

7. FAO/WHO. 1996. Biotechnology and Food Safety, Report of a Joint FAO/WHO Consultation. FAO Food and Nutrition Paper 61. Rome, Italy.

8. Jonas, D.A., Antignac, E., Antoine, J.M., Classen, H.G., Huggett, A., Knudsen, I., Mahler, J., Ockhuizen, T., Smith, M., Teuber, M., Walker, R., and de Vogel, P. 1996. The Safety Assessment of Novel Foods, Guidelines prepared by ILSI Europe Novel Food Task Force. Food Chem. Toxicol. 34: 931-940.

9. 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.

10. 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.

GENENCOR

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

12 . IUBMB. Enzyme nomenclature. Recommendations 1992. http://www.chem.qmw.ac.uk/iubmb/enzyme; 2001.

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

14 . 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.

15 . 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.

16 . U.S. Pharmacopeia. 2010. Monograph: Enzyme Preparations. In: Food Chemicals Codex, *7th Edition*. Washington, D.C, pp. 323-327.

17 . JECFA 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.

18. White PJ and Johnson LA, 2003. "Corn Sweeteners," in Corn Chemistry and Technology, 2nd Edition

19. Alexander RJ, 1998. "Production and Description," in Sweeteners: Nutritive.

20. Corn Refiners Association, 2006. "Manufacture," in Nutritive Sweeteners from Corn, 8th Edition.

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

22. 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.

23. 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.

24. 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.

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

26. 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.

27. 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.

28. 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.

29. 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

30. 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

31. 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.

32. Ivanciuc O, Schein CH, and Braun W. 2003. SDAP: Database and Computational Tools for Allergenic Proteins. Nucleic Acids Res. 31: 359-362.

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

34. 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.

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

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

37. Internatinoal Union of Immunological Societies (http://www.allergen.org/)

38. 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

39. GRAS Notice 90. 2001. Carbohydrase enzyme preparation from *Aspergillus oryzae*, protease enzyme preparation from *Aspergillus oryzae*, and carbohydrase enzyme preparation from *Rhizopus oryzae* http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=90

40. Code of Federal Regulations Title 21. Part 173. Sec. 173.130 Carbohydrase derived from *Rhizopus oryzae.*

GENENCOR

10. LIST OF APPENDICES

Appendix 1 – A) The amino acid sequence of *T. reesei* GA
B) Similarity table of *T. reesei* glucoamylase and 10 other GA isolates on Protein sequence level

Appendix 2 -- The Manufacturing process

Appendix 3 – *T. reesei* Strain Lineage and Summary of Safety Studies

Appendix 4 -- Composition of Tox Lot

Appendix 5 – Analysis of Safety based on Pariza/Johnson Decision Tree

Appendix 6 – GRAS Concurrence letter

GENENCOR

Appendix 1A – The amino acid sequence of *T. reesei* Glucoamylase

In FASTA format, without the secretion signal.

PGSSGLSDVTKRSVDDFISTETPIALNNLLCNVGPDGCRAFGTSAGAVIASPSTIDPDYYYMWTR
DSALVFKNLIDRFTETYDAGLQRRIEQYITAQVTLQGLSNPSGSLADGSGLGEPKFELTLKPFTG
NWGRPQRDGPALRAIALIGYSKWLINNNYQSTVSNVIWPIVRNDLNYVAQYWNQTGFDLWEEVNG
SSFFTVANQHRALVEGATLAATLGQSGSAYSSVAPQVLCFLQRFWVSSGGYVDSNINTNEGRTGK
DVNSVLTSIHTFDPNLGCDAGTFQPCSDKALSNLKVVVDSFRSIYGVNKGIPAGAAVAIGRYAED
VYYNGNPWYLATFAAAEQLYDAIYVWKKTGSITVTATSLAFFQELVPGVTAGTYSSSSSTFTNII
NAVSTYADGFLSEAAKYVPADGSLAEQFDRNSGTPLSALHLTWSYASFLTATARRAGIVPPSWAN
SSASTIPSTCSGASVVGSYSRPTATSFPPSQTPKPGVPSGTPYTPLPCATPTSVAVTFHELVSTQ
FGQTVKVAGNAAALGNWSTSAAVALDAVNYADNHPLWIGTVNLEAGDVVEYKYINVGQDGSVTWE
SDPNHTYTVPAVACVTQVVKEDTWQS

GENENCOR

Appendix 1B

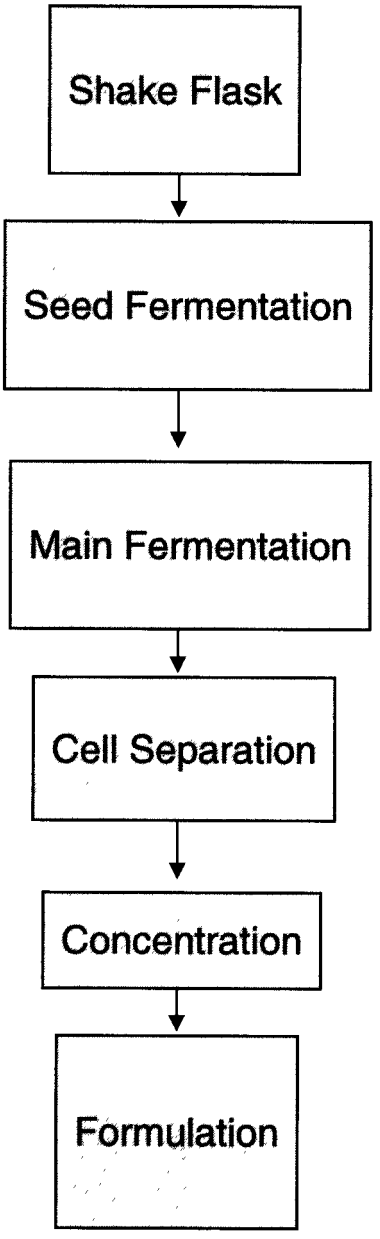
Similarity table of *T. reesei* glucoamylase and 10 other GA isolates on Protein sequence level (original data analysis reported 07/06/06, verified 05/10/10)

	A.niger GA CD	*T.emersonii*	*A.oryzae*	*P.chrysogenum*	*H.resinae*	*G.zeae* PH1-10	*G.zeae* PH1-12	*T.reesei*	*H.grisea*	*N.crassa*	*M.grisea*
A.niger	100	66	60	61	52	56	52	56	53	61	60
T.emersonii	66	100	60	61	58	57	53	61	56	61	59
A.oryzae	60		100	68	54	56	50	58	54	60	57
P.chrysogenum	61			100	56	56	51	57	54	62	56
H.resinae	52				100	51	47	55	51	54	53
G.zeae PH1-10	56					100	52	57	55	60	54
G.zeae PH1-12	52						100	59	59	57	57
T.reesei	56							100	62	68	60
H.grisea	53								100	64	58
N.crassa	61									100	60
M.grisea	60										100

A. niger = *Aspergillus niger*
T. emersonii = *Talaromyces emersonii*
A. oryzae = *Aspergillus oryzae*
P. chrysogenum = *Penicillium chrysogenum*
H. resinae = *Hormoconis resinae*
G. zeae = *Giberella zeae*
T. reesei = *Trichoderma reesei*
H. grisea = *Humicola grisea*
N. crassa = *Neurospora crassa*
M. grisea = *Magnaporthe grisea*

GENENCOR

Appendix 2- The Manufacturing Process

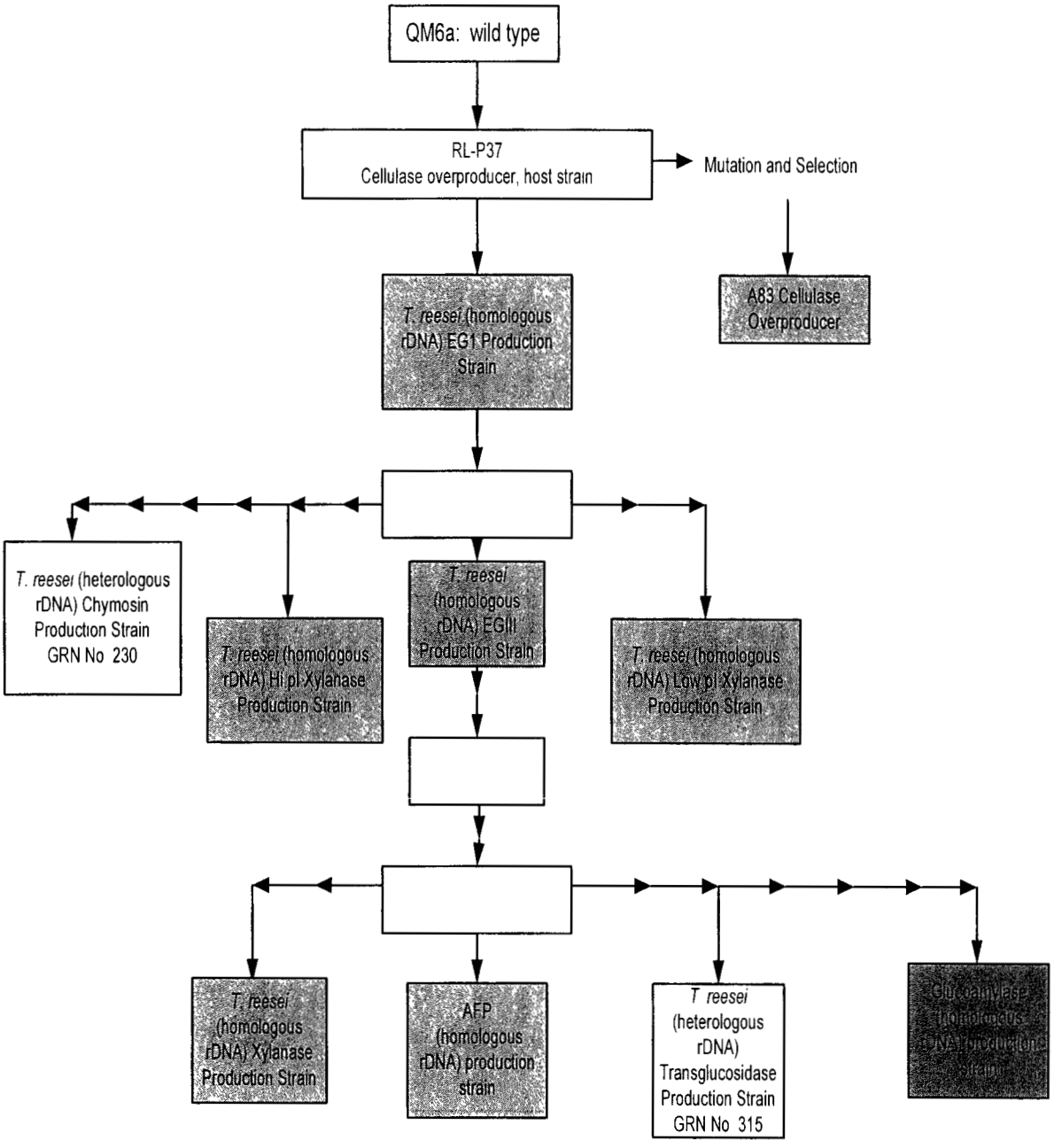


GENENCOR

Appendix 3 – *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 green color indicates the strain is the subject of this submission.



BEST ORIGINAL COPY

GENENCOR

Toxicology Test Summaries

The safety of the six enzyme preparations derived from the six 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 the non-recombinant strain *T. reesei* A83 and product derived from it.

PRODUCTION ORGANISM	ENZYME PREPARATION	TOXICOLOGY TEST	RESULT
I. *T. reesei* A83 (traditionally modified)	*T. reesei* cellulase	Pathogenicity study, rats	Non-pathogenic, non-toxicogenic
		91-day subchronic study, rats	No adverse effect detected
		Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosome assay, human lymphocytes	Not clastogenic
II. *T. reesei* (homologous rDNA)	High pI Xylanase	91-day subchronic study, rats	No adverse effects detected
		Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosome assay, Chinese hamster ovary cells	Not clastogenic
III. *T. reesei* (homologous rDNA)	Low pI Xylanase	91-day subchronic study, rats	No adverse effects detected
		Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosome assay, human lymphocytes	Not clastogenic
IV. *T. reesei* (homologous rDNA)	Endoglucanase III	28-day subacute study, rats	No adverse effects detected
		Bacterial reverse mutation assay	Not mutagenic

GENENCOR

PRODUCTION ORGANISM	ENZYME PREPARATION	TOXICOLOGY TEST	RESULT
V. *T. reesei* (homologous rDNA)	Endoglucanase I	14-day subacute study, rats	No adverse effects detected
		Pathogenicity study, rats	Non pathogenic
		91-day subchronic study, rats	No adverse effects detected NOAEL = 1000 mg/kg/d
		In vitro chromosome assay, human lymphocytes	Not clastogenic
VI. *T. reesei* (homologous rDNA)	Xylanase (protein engineered)	91-day subchronic study, rats	No adverse effects detected
		Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosome assay, human lymphocytes	Not clastogenic
VII. *T. reesei* (homologous rDNA)	Acid Fungal Protease	Acute dermal irritation in rabbits	Not a skin irritant
		Acute oral toxicity in rats	Non-hazardous (oral LD_{50} > 2000 mg/kg)
		Ocular irritation in rabbits	Not an eye irritant
		Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosome assay, human lymphocytes	Not clastogenic
		91-day subchronic study, rats	No adverse effects detected

All enzyme preparations were found to be non-toxic, non-mutagenic and not clastogenic.

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 0 (control), 1, 2, or 5% w/w 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 up to and including a dose level of 5% w/w in the diet. A NOEL (No Observed Effect Level) was established at 5% in the diet corresponding to 3.35 and 4.05 grams of *T. reesei* cellulase/kg body weight/day for male and female rats, respectively.

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. 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 0 (control), 10000, 20000, or 50000 ppm (expressed in terms of total organic solids) 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 a dose level of 50000 ppm (expressed in terms of TOS) in the diet. A NOEL (No Observed Effect Level) was established at 50000 ppm in the diet.

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 (Corning Hazleton, 1995). 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* 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 (Corning Hazleton, 1995). 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.

III. 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 0 (control), 750, 1500 or 3000 mg/kg/day 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 a dose level of 3000 mg/kg/day. A NOEL (No Observed Effect Level) was established at 3000 mg/kg/day for male and female rats, respectively.

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.

IV. 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 (EG III) was given by gavage to groups of male and female Charles River rats at 0 (deionized water), 40, 200 or 1000 mg/kg/day 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 *T. reesei* EG III. 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 (No Observed Effect Level) was established at 1000 mg/kg/day.

B. Bacterial reverse mutation assay (Ames assay)

The test article, EG III, 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, EG III was not mutagenic in the bacterial reverse mutation assay using *Salmonella* and *E. coli*.

V. 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* strain EG1-EP9 was tested in male and female CD rats following an acute intraperitoneal injection of 5.6 x 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* strain EG1-EP9 was 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 (EGI) 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 0 (deionized water), 40, 200 or 1000 mg/kg/day for 28 consecutive days (IRDC, 1993). There were no adverse effects on any parameter monitored in this study for rats treated with EGI. It was concluded that the treatment of male and female rats with EGI 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 250, 500 or 1000 mg/kg/day 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 EGI from *T. reesei* did not result in toxicity up to and including a dose level of 1000 mg/kg/day. A NOEL (No Observed Effect Level) was established at 1,000 mg/kg/day.

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

Endoglucanase I (EGI) 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, 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 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.

VI. 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 5000, 12500 or 37500 RBB U/kg/day 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 a dose level of 37500 RBB U/kg/day. A NOEL (No Observed Effect Level) was established at 37500 RBB U/kg/day.

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.

VII. Acid Fungal Protease (AFP) from *T. reesei* (homologous rDNA)

A. Ocular Irritation in the Rabbits

In the initial study, slight conjunctivitis was observed at the 1-hour observation period with clearing by 24 hours. In the confirmatory assay, no irritation was observed.

The primary eye irritation score was 0.0.

B. Acute dermal irritation study in rabbits (sequential approach)

No deaths or overt signs of toxicity were observed in this study. No effects on feed consumption and weight gain were recorded. No reactions were noted at any test site in both preliminary and confirmatory assays.

The mean score for skin edema and erythema was 0.0. According to the Directive of the Commission 93/21/EEC of April 27, 1993, AFP is not a skin irritant.

C. Acute oral toxicity in rats

No mortality was recorded in this study. There were no treatment related effects noted throughout the 14-day observation period.

Using the GHS classification system (December 2001), AFP can be classified as non-hazardous (oral LD_{50} > 2000 mg/kg bw).

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

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 (no observed adverse effect level) is established at the highest dose tested (31.25 mg total protein/kg bw/day or 35.81 mg TOS/kg bw/day).

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

In the preliminary dose-range assay, cytotoxicity was observed at 5000 μg/ml in both non-activated and activated cultures. Based on these results, the following doses were used:

First main test, without S-9 mix: 625, 1250 and 2500 µg/ml
First main test, with S-9 mix: 1250, 2500 and 5000 µg/ml
Second main test, without S-9 mix: 313, 625 and 1250 µg/ml
Second main test, with S-9 mix: 1250, 2500 and 5000 µg/ml

In the absence of S-9 mix, AFP caused reductions in mean mitotic index of 52 and 53% at the highest concentration tested in the first and second main test, respectively, and this level of toxicity meets the requirements of OECD 473 guideline for the highest concentration to be scored for aberrations (> 50% reduction in mitotic index). In the presence of S-9 mix, the 5000 µg/ml dose level is the maximum required by OECD guideline.

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, AFP concentrate did not induce chromosomal aberrations (both structural and numerical) in mammalian cells both in the presence and absence of metabolic activation.

F. Bacterial Reverse Mutation Assay – Ames assay

In the preliminary toxicity test, AFP was not toxic to the bacteria at any dose level tested. Therefore, the highest dose level selected for the main assays was 5000 µg/plate, which is the maximum required by OECD guideline.

In the first main test, AFP concentrate was not toxic to tester strains at any dose level, either in the presence or absence of S-9 mix. In the second main test, a statistically significant reduction in the number of revertant colonies was observed in strain TA 1535 with S-9 mix, but this effect was not dose related.

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.

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, AFP has not shown any evidence of mutagenic activity in the Ames assay.

GENENCOR

Appendix 4 – Certificate of Analysis of Tox Lot

Genencor International®

925 Page Mill Road · Palo Alto, CA 94304-1013 USA · 650 846-5866 tel · 650 845-6505 fax · www.genencor.com

CERTIFICATE OF ANALYSIS

Name of Test Article: **Glucose Amylase (TrGA)**

Production/Strain Name: *Trichoderma reesei* 70H2-TrGA#32-9

Production Site: Beloit, USA

Genencor International Culture Collection Number: GICC 03306

Designation of Lot Tested: trg08050/52

Description: Clear brown liquid

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: 735 GAU/g

2. Total and TCA Protein
   The samples were measured for TCA and total protein by nitrogen analysis (with a conversion factor of 5.86 g protein/g nitrogen).

   Total Proteins: 199.63 mg/ml
   TCA Proteins: 147.86 mg/ml
   % Total Organic Solids: 20.73%
   (100% – moisture% – ash%)

3. Specific gravity: 1.07 g/ml

4. pH: 3.80

GENENCOR

5. Microbial analysis: Microbial analysis conducted by GCOR, Rochester, NY

Analysis	Results
Total viable count	< 10 CFU/ml
Coliform	< 1CFU/ml
E. Coli	negative/25 ml
Salmonella	negative/25 ml
Staphylococcus aureus	< 1 CFU/ml
Pseudomonas	< 1 CFU/ml
Production strain	negative

6. Mycotoxin analysis: Negative

7. Heavy metals analysis (analysis by ICP-OES/DMA)

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

8. Stability Data

Room Temperature (all activity units are reported in g)

Sample ID	Dilution	T = 0	T = 5 hours	% of T = 0
Trg 08050/52	straight	727	719	98.9
Trg 08050/52	1/2	366	364	99.4
Trg 08050/52	1/4	191	188	98.4

Refrigerator (4C): Undiluted Material

Sample ID	Dilution	T=0	T = 7 days	% of T = 0
Trg 08050/52	straight	735	732	99.6

Frozen (-20°C) : Undiluted Material

Sample ID	Dilution	T = 0	T = 30 days	% of T = 0	T = 90 days	% of T = 0
Trg 08050/52	straight	735	690	93.9	724	98.5

GENENCOR

Study Sponsor Bio-Analytical Representatives:

(b) (6) .....Date: 1/29/09
Annette Diaz

(b) (6) ..... Date: 1/29/2009
Angela Shickles

Study Sponsor Monitor
(b) (6)
.....Date: January 28, 2009
Quang Q. Bui

GENENCOR

Appendix 5 -Analysis of Safety Based on Pariza/Johnson Decision Tree

Pariza and Johnson have published guidelines for the safety assessment of microbial enzyme preparations (2001). These guidelines are based upon decades of experience in the production, use and safety evaluation of enzyme preparations. The safety assessment of a given enzyme preparation is based upon an evaluation of the toxigenic potential of the production organism. The responses below follow the pathway indicated in the decision tree. The outcome of this inquiry is that this Glucoamylase enzyme preparation is "ACCEPTED" as safe for its intended use.

1. Is the production strain genetically modified? 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 have a history of safe use in food? Yes. Glucoamylase has been used for years in food processing. The *Trichoderma reesei* glucoamylase is new as an isolate in food processing, but has been present at lower amounts in other enzyme preparations from this host. It is also listed in the Brewer's Adjunct Manual. Go to 3c.

3c. Is the test article free of transferable antibiotic resistance gene DNA? Yes, no antibiotic resistance genes were used in the construction. Go to 3e.

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

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

5. Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed? Yes. The inserted DNA is well characterized. Complete characterization of the location of insertion is not possible. 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? Yes. The *T. reesei* RL-P37 safe lineage is established as discussed in Appendix 3. Its safety as a production host and methods of modification are well documented, and their safety have been confirmed through repeated toxicology testing; Genencor has conducted toxicology testing on 8 different products derived from this lineage, including this one, and all were determined to be safe for their intended food uses; Accept.

Conclusion: Article is accepted

GENENCOR

Appendix 6 – GRAS Concurrence Letter from Dr. Michael W. Pariza

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

Michael W. Pariza, Member

December 28, 2010

Vincent J. Sewalt, PhD
Director of Regulatory Affairs
Danisco US Inc
925 Page Mill Road
Palo Alto, CA 94304

Dear Dr. Sewalt,

I have reviewed the new information that you provided on Danisco's *Trichoderma reesei* glucoamylase enzyme preparation, which is produced utilizing an improved strain of *T. reesei*, designated *T. reesei* 70H2-TrGA #32-9. The intended uses of the enzyme are in grain processing including the manufacture of corn sweeteners such as high fructose corn syrup, brewing, potable alcohol, and lactic acid for both food and feed.

In evaluating Danisco's glucoamylase enzyme preparation, I considered the biology of *Trichoderma reesei*, relevant information available in the peer-reviewed scientific literature, and information that you provided regarding the cloning methodology that was utilized, the safe lineage of the production organism, and results of the safety evaluation studies that Danisco performed.

By way of background, *T. reesei* is a non-pathogenic, non-toxigenic fungus that 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. Danisco's new production organism, *T. reesei* 70H2-TrGA #32-9, was derived by a series of modifications from *T. reesei* QM6a, the original wild-type parental strain used by Danisco to produce a safe strain lineage. Published literature, government laws and regulations, for example FR 64:28658-28362 (1999), reviews by expert panels such as FAO/WHO JECFA (1992), and Danisco's unpublished safety

studies, all support the conclusion that the lineage to which *T. reesei* 70H2-TrGA #32-9 belongs 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, cellulase, glucoamylase, β-glucosidase/cellulase, alpha-amylase, acid fungal protease, transglucosidase, and phytase. The enzymes from these strains, and in two cases the strains themselves, have been subjected to 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 and 00315, both of which carry the decision statement, "FDA has no questions."

Danisco's glucoamylase enzyme preparation, produced utilizing *T. reesei* 70H2-TrGA #32-9, was evaluated in a 13-week subchronic feeding trial in rats, an acute (14 day) toxicity study in rats, tests for acute dermal and ocular irritation in rabbits, and a bank of genetic toxicology tests. No adverse effects were observed. The glucoamylase enzyme preparation was assessed for allergenic potential using methods developed by FAO/WHO, which involve searching the primary enzyme structure for regions that match known allergens. No suspect regions were observed. Finally, Danisco has developed manufacturing conditions and specifications for the glucoamylase enzyme preparation that are appropriate and suitable for a food-grade ingredient.

Human exposure estimates to *T. reesei* 70H2-TrGA #32-9 protein residues from the intended uses of the Danisco glucoamylase enzyme preparation in grain processing including the manufacture of corn sweeteners such as high fructose corn syrup, brewing, potable alcohol, and lactic acid, are well within the generally-accepted (100-fold below NOAEL) safety limit. In addition, there are no safety concerns to target animal species from the presence of *T. reesei* 70H2-TrGA #32-9 protein residues in feed-grade lactic acid manufactured using the Danisco glucoamylase enzyme preparation.

The safety of the glucoamylase enzyme preparation was formally evaluated using the Pariza-Johnson decision tree. The conclusion of this analysis was that the test article (enzyme preparation) was accepted.

Based on the forgoing, I concur with the evaluation made by Danisco, that the *Trichoderma reesei* 70H2-TrGA #32-9 production strain is safe to use for the manufacture of food grade glucoamylase. I further conclude that the glucoamylase enzyme preparation manufactured using this production strain by the process you described is GRAS (Generally Recognized As Safe) for use as in grain processing including

GENENCOR

the manufacture of corn sweeteners such as high fructose corn syrup, brewing, potable alcohol, and lactic acid for both food and feed. 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
University of Wisconsin-Madison

GENENCOR

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

February 16, 2011

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

RECEIVED
FEB 18 2011
Division of Biotechnology and GRAS Notice Review

RE: GRAS Notification – Availability of Information Statement

Dear Dr. Gaynor,

Enclosed are three copies of the corrected "Availability of Information" statement.

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

Sincerely,

(b) (6)

Vincent Sewalt, PhD
Director of Regulatory Affairs
Genencor, a Danisco Division
650-846-5861 / vincent.sewalt@danisco.com

Enclosures

GENENCOR

1.1 Exemption from Pre-market Approval

Pursuant to the regulatory and scientific procedures established in proposed 21 C.F.R. 170.36, Genencor, a Danisco Division, ("Danisco") has determined that its glucoamylases (GA) enzyme preparation produced by *Trichoderma reesei* expressing the gene encoding GA from *T. reesei* is a Generally Recognized as Safe ("GRAS") substance for the intended food application and is, therefore, exempt from the requirement for premarket approval.

1.2 Name and Address of Notifier

Genencor, A Danisco Division
925 Page Mill Road
Palo Alto, CA 94304

1.3 Common or Usual Name of Substance

Glucoamylase (GA) enzyme preparation from *Trichoderma reesei* expressing the gene encoding the GA from *T. reesei* (TrGA).

1.4 Applicable Conditions of Use

Glucoamylase is used as a processing aid in grain processing, including the manufacture of corn sweeteners such as high fructose corn syrup (HFCS), brewing, potable alcohol manufacture, and lactic acid production.

1.5 Basis for GRAS Determination

This GRAS determination is based upon scientific procedures.

1.6 Availability of Information for FDA Review

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

Submission End