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December 4, 2009

Dr. Robert Martin
Deputy Division Director
Office of Food Additive Safety (HFS-255)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5 100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Dr. Martin:

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 transglucosidase enzyme preparation produced by *Trichoderma reesei* expressing the gene encoding transglucosidase from *Aspergillus niger* is a Generally Recognized as Safe ("GRAS") substance for the intended food application and is, therefore, exempt from the requirement for premarket approval. We are hereby submitting, in triplicate, a Generally Recognized As Safe ("GRAS") Notification, in accordance with proposed 21 C.F.R. § 170.36, informing FDA of the view of Danisco that the transglucosidase enzyme preparation is GRAS, through scientific procedures, for conversion of the malto-oligosaccharides in starch from grains to isomalto-oligosaccharides (IMO) and as one of the enzymes in an enzyme cocktail used to produce potable alcohol from molasses. In these applications the enzyme is considered a processing aid.

If you have questions or require additional information, please contact me at 650-846-7557 or fax at 650-845-6505.

Sincerely,

(b) (6)

Alice J. Caddow
Vice President of Regulatory
And Environmental Affairs

Enclosures (3 binders)

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Transglucosidase Enzyme Preparation Derived from

Trichoderma reesei

Expressing the Transglucosidase Gene

from

Aspergillus niger Is Generally Recognized As Safe

For Use in Food Processing

Notification Submitted by Genencor, a Danisco Division

December 4, 2009

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1. GENERAL INTRODUCTION

The transglucosidase (TG) enzyme preparation under consideration is produced by submerged fermentation of *Trichoderma reesei* carrying the gene encoding the transglucosidase enzyme from *Aspergillus niger*. This commercial TG product will replace the current *A. niger* derived TG.

Use of the enzyme product is intended to convert the malto-oligosaccharides in starch from grains to isomalto-oligosaccharides (IMO) and as one of the enzymes in an enzyme cocktail used to produce potable alcohol from molasses. In these applications, the *Trichoderma* transglucosidase will primarily be replacing transglucosidase from the *A. niger* commercial sources. In this application, transglucosidase will be used as a processing aid where the enzyme is not present in the final food.

The systematic name of the principle enzyme activity is 1,4-α-D-glucan:1,4-α-D-glucan (D-glucose) 6-α-D-glucosyltransferase. Other names used are Oligoglucan-branching glycosyltransferase; 1,4-α-D-glucan 6-α-D-glucosyltransferase; T-enzyme; D-glucosyltransferase, and transglucosidase.

The enzyme catalyses both hydrolytic and transfer reactions on incubation with α-D-gluco-oligosaccharides. Transfer occurs most frequently to HO-6, producing isomaltose from D-glucose, and panose from maltose. Transglucosidase can also transfer to the HO-2 or HO-3 of D-glucose to form kojibiose or nigerose, or back to HO-4 to form maltose. The action on maltose produces equimolar concentration of panose and glucose.

The EC number of the enzyme is 2.4.1.24, and the CAS number is 9030-12-0.

Pursuant to the regulatory and scientific procedures established in proposed 21 C.F.R. 170.36, Danisco has determined that its TG enzyme preparation from a modified strain of *T. reesei* expressing the transglucosidase enzyme from *Aspergillus niger* is a Generally Recognized As Safe ("GRAS") substance for the intended food application and is, therefore, exempt from the requirement for premarket approval. The information provided in the following sections is the basis of our determination of GRAS status of this TG 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 for this TG, *T. reesei,* 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.[3-8] 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.

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1.1 Name and Address of Notifier

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

1.2 Common or Usual Name of Substance

Transglucosidase enzyme preparation from *Trichoderma reesei* expressing the gene encoding the transglucosidase enzyme from *Aspergillus niger*

1.3 Applicable Conditions of Use

Transglucosidase is used as a processing aid in the production of IMO syrups from starch and in the production of potable ethanol from molasses. To our knowledge, use of transglucosidase is the only method of producing IMOs from starch.

1.4 Basis for GRAS Determination

This GRAS determination is based upon scientific procedures.

1.5 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. 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.

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The production organism is a strain of *Trichoderma reesei*, which has been genetically modified by deletion of several cellulase genes and to express a gene for the production and secretion of transglucosidase isolated from *A. niger* (see Section 2.2). *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]. It contains the transglucosidase gene under the regulation of a native *T. reesei cbhI* (cellobiohydrolase 1) gene. The *A. nidulans amdS* gene

was used as a selectable marker. The inserted DNA was integrated into the recipient chromosome.

2.2 Host Microorganism

The host organism *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 *Trichoderma 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 Transglucosidase Expression Vector

For construction of the vector, the DNA encoding the *A. niger* mature secreted transglucosidase protein was fused to the *T. reesei* CBHI signal peptide. This open reading frame is flanked by the promoter and terminator sequences of the *T. reesei cbh1* gene. The vector also contains the *Aspergillus nidulans amdS* gene, encoding acetamidase, as a selectable marker for transformation of *T. reesei.*

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 that only the intended genetic modifications to the *T. reesei* strain had been made.

2.4 Stability of the Introduced Genetic Sequences

The production strain proved to be 100% stable after at least 60 generations of fermentation, judged by transglucosidase production.

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 1CFU/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.

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3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1 Enzyme Identity

IUBMB Nomenclature[12]:	1,4-α-glucan-6-α-glucosyltransferase
IUBMB Number:	2.4.1.24
CAS Number:	9030-12-0
Reaction catalyzed:	Transglucosidase transfers an α-D-glucosyl residue in a 1,4-α-D-glucan to the primary hydroxyl group of glucose, free or combined in a 1,4-α-D-glucan.
Other Names:	Oligoglucan-branching glycosyltransferase; 1,4-α-D-glucan 6-α-D-glucosyltransferase; T-enzyme; D-glucosyltransferase[12].
Systematic Name:	1,4-α-D-glucan:1,4-α-D-glucan (D-glucose) 6-α-D-glucosyltransferase[12].

Technical Effect:
Transglucosidase catalyses both hydrolytic and transfer reactions on incubation with α-D-gluco-oligosaccharides. Transfer occurs most frequently to HO-6, producing isomaltose from D-glucose, and panose from maltose. Transglucosidase can also transfer to the HO-2 or HO-3 of D-glucose to form kojibiose or nigerose, or back to HO-4 to form maltose. The action on maltose produces equimolar concentration of panose and glucose.

As the result of transglucosidase reactions, the malto-oligosaccharides are converted to isomalto-oligosaccharides containing high proportions of glucosyl residues linked by an α-D-1,6 linkage from the non-reducing end. In molasses, non-fermentable sugars including raffinose and stachyose are converted to sucrose, galactose, glucose and fructose, which can then be fermented into alcohol.

3.2 Amino Acid Sequence

The amino acid sequence of the TG enzyme and nucleotide sequence of the gene encoding it are freely available from public databases such as GenBank (Accession numbers BAA23616, D45356). The sequence corresponding to the mature transglucosidase (between the positions 1251 and 1252 in the GenBank sequence D45356,[33,34] was used for this construction.

The amino acid sequence of the TG enzyme is shown in Appendix 1.

4. MANUFACTURING PROCESS

This section describes the manufacturing process for this TG enzyme which follows standard industry practice.[23-25] 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 TG concentrate are standard ingredients used in the enzyme industry.[23-25] All the raw materials conform to the specifications of the Food Chemicals Codex, 6th edition, 2008 ("FCC"),[14] 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 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 and flocculants used in the fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 24, 1998. The maximum use level of these antifoam in the production process is $\leq$0.15% and of the flocculants <1.5%.

4.2 Fermentation Process

The TG enzyme is manufactured by submerged straight-batch or fed-batch pure culture fermentation of the genetically modified strain of *T. reesei* described in Section 2. All equipment is carefully designed, constructed, operated, cleaned and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are conducted periodically to ensure absence of foreign microorganisms and confirm production strain identity.

4.2.1 Production organism

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

4.2.2 Criteria for the rejection of fermentation batches

Growth characteristics during fermentation are observed microscopically. Samples are taken from each fermentation stage (inoculum, seed-, and main fermentor) before inoculation, at regular intervals during growth and before harvest or transfer. These samples are tested for microbiological contamination by plating on a nutrient medium.

A fermentation batch is declared as 'contaminated' if colony forming units (CFU) of bacteria or fungi other than the production strain are present at levels >10^3CFU/ml.

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. Polish filtration – for removal of residual production strain organisms and as a general precaution against microbial degradation
3. Concentration – ultrafiltration
4. Formulation.

4.3.2 Formulation and standardization process

The ultrafiltered concentrate is stabilized with 3 % sodium chloride and 50% glycerol.

4.3.3 Quality control of finished product

The final TG 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")[15] in 2006 and the FCC[14]. These specifications are set forth in Section 5.

5. COMPOSITION AND SPECIFICATIONS

5.1 Quantitative Composition

The liquid concentrate is stabilized with 3 % sodium chloride and 50% glycerol and tested to demonstrate that it meets the specifications in section 5.2.

The formulation of the enzyme preparation is as follows:

- 3 % enzyme protein
- 3 % sodium chloride
- 50% glycerol
- 44% water.

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5.2 Specifications

Transglucosidase meets the purity specifications for enzyme preparations set forth in Food Chemicals Codex[14]. In addition, it also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications [15].

6. APPLICATION

6.1 Mode of Action

Transglucosidase catalyses both hydrolytic and transfer reactions on incubation with α-D-gluco-oligosaccharides. Transfer occurs most frequently to HO-6, producing isomaltose from D-glucose, and panose from maltose. Transglucosidase can also transfer to the HO-2 or HO-3 of D-glucose to form kojibiose or nigerose, or back to HO-4 to form maltose. The action on maltose produces equimolar concentrations of panose and glucose.

As the result of transglucosidase reactions, the malto-oligosaccharides are converted to isomalto-oligosaccharides containing high proportions of glucosyl residues linked by an α-D-1,6 linkage from the non-reducing end. In molasses, non-fermentable sugars including raffinose and stachyose are converted to sucrose, galactose, glucose and fructose, which can then be fermented into alcohol.

6.2 Use Levels

Transglucosidase is used as a processing aid in the production of IMO syrups from starch and in the production of potable ethanol from molasses. To our knowledge, use of transglucosidase is the only method of producing IMOs from starch.

IMO
The dose of TG product for IMO production is 0.5 to 1.5 kg per ton of DS. After about two days' reaction, the enzyme will be deactivated at about 80°C. Followed by carbon treatment for decoloration, then ion exchange, so there should be no enzyme left in the end product similar to other saccharifying enzymes for glucose or maltose production.

Potable Ethanol
The enzyme product dose is 6-20 ppm, added during fermentation. After about 10 to 24 hours fermentation, the broth will go through distillation, where all the enzyme will be deactivated.

6.3 Enzyme Residues in the Final Foods

As discussed above, the enzyme will be deactivated in both applications and will not be present in the final food products.

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[1] 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 that *T. reesei* be placed into synonymy with *T. longibrachiatum*[13]. Later however, evidence emerged indicating that the two species are not identical[9]. 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[17]. Their work was confirmed by another group that found evidence of peptaibol production in two other *T. reesei* strains[18]. 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 the FCC.

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.[19], Blumenthal[20] and Olemska-Beer et al.[21]. 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 various strains have been approved to produce commercial enzyme products internationally, for example, in Canada (Food and Drugs Act Division 16, Table V, Food Additives That May Be Used As Enzymes), the United States (FDA 1999), France, Australia/New Zealand, China (MOH 1996), and Japan.

The production organism of the transglucosidase enzyme preparation, the subject of this submission, is *T. reesei* strain TG #626, GICC 03289. It is derived by recombinant DNA methods from strain RL-P37. The purpose of this genetic modification is to express the transglucosidase from *A. niger* in *T. reesei*. RL-P37, a commercial production strain, is derived, as a result of 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 strain has been determined to be non-pathogenic and non-toxicogenic through an acute intraperitoneal study in rats. All of the food/feed grade products produced by this lineage were determined to be safe for their intended uses and are the subject of numerous GRAS determinations. A GRAS Notice was filed for one of the products from this strain lineage and FDA issued a no questions letter (see GRAS Notice 230[42]).

From the information reviewed, it is concluded that the organism *T. reesei* provides no specific risks to human health and is safe to use as the production organism of transglucosidase.

It is concluded that the strain is non-pathogenic and non-toxigenic.

7.1.2 Safety of the donor source

Aspergillus niger has a long history of safe use in the production of industrial enzymes and chemicals of both food grade and technical grade. It is one of the most important producers of industrial neutral proteases[22] and glucoamylases[23]. The species is listed as a

production/donor organism for a series of food-grade carbohydrases, oxidoreductases, lipases, glucanotransferase, and proteases in the recent Pariza and Johnson paper[1]. Several *A. niger*-derived substances used in the food industry have acquired GRAS status for the proposed applications:

- Carbohydrase and cellulase enzyme preparation (21 CFR §173.120)[26];
- Citric acid (21 CFR §173.280)[27];
- Carbohydrase enzyme preparation, catalase enzyme preparation, glucose oxidase enzyme preparation, pectinase enzyme preparation, and protease enzyme preparation (GRSN 000089)[28]; and
- Lactase (GRSN 0000132); Lipase (GRSN 0000111), Asparaginase (GRSN 000214), Phosplipase A2 (GRSN 0000183)[29].

The safety of *A. niger* was summarized by Schuster *et al*[30] and was also discussed by Olempska-beer *et al*[21].

In 1997, *A. niger* became one of the ten microbial species/strains that were eligible for exemption under 40 CFR Part 725 as recipient microorganisms under the TSCA biotechnology regulations[31], based on EPA's risk assessments.

The donor organism for the transglucosidase gene used in construction of the new microorganism *T. reesei* TG #626, GICC 03289 was *Aspergillus niger* AGME 9, also known as SE4, and described in US patent 5,783,414. *A. niger* AGME9 is the source of Genencor's current transglucosidase product. The enzyme from *A. niger* is one of the well known forms of transglucosidases[32]. The amino acid sequence of the enzyme and nucleotide sequence of the gene encoding it are freely available from public databases such as GenBank (Accession numbers BAA23616, D45356). The sequence corresponding to the mature transglucosidase (between the positions 1251 and 1252 in the GenBank sequence D45356,[33,34] was used for this construction.

The *A. niger* transglucosidase is GRAS as previously determined by Genencor and concurred with by Dr. Michael Pariza. Additionally, it is described as the enzyme used to produce isomalto-oligosaccharides in GRAS Notice 000246 and the corresponding FDA no-questions letter[35].

7.2 Safety of the Manufacturing Process

The manufacturing process for the production of transglucosidase will be conducted in a manner similar to other food and feed production processes. It consists of a pure-culture fermentation process, cell separation, concentration and formulation, resulting in a liquid transglucosidase 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, 6th Edition[14] and the general specifications for enzyme preparations used in food processing proposed by WHO/JECFA[15].

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7.3 Safety of Transglucosidase

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. Transglucosidases have been used in food processes to generate IMOs for several years and have generated no known safety concerns.

In 1998 the Association of Manufacturers of Fermentation Enzyme Products (AMFEP) Working Group on Consumer Allergy Risk from Enzyme Residues in Food reported on an in-depth analysis of the allergenicity of enzyme products. They concluded that there are no scientific indications that small amounts of enzymes in bread and other foods can sensitize or induce allergy reactions in consumers, and that enzyme residues in bread and other foods do not represent any unacceptable risk to consumers. Further, in a recent investigation of possible oral allergenicity of 19 commercial enzymes used in the food industry, there were no findings of clinical relevance even in individuals with inhalation allergies to the same enzymes, and the authors concluded "that ingestion of food enzymes in general is not considered to be a concern with regard to food allergy[36]."

Despite this lack of general concern, the potential that transglucosidase could be a food allergen was assessed by comparison with sequences of known allergens and is summarized here. FASTA alignments of the *A. niger* TG sequence with known allergens using both the SDAP database (http://fermi.utmb.edu/SDAP/sdap_who.html)containing 887 allergens (listed in http://fermi.utmb.edu/cgi-bin/SDAP/sdap_01) or the combined SwissProt and WHO-IUIS databases in Allermatch (www.allermatch.org) containing 792 allergens reveals no significant (E<0.01) homology with any known allergens.

Following the Codex (2003)[37] guidelines, homology searches were performed against the combined Allermatch database as well as the SDAP database, using a sliding window of 80-amino acids and greater than 35% identity to indicate a match, which revealed no matches of *A. niger* TG with any known allergens. This Codex recommended search is most authoritative as it reduces the likelihood of false negatives or false positives[38,39].

A secondary, more controversial search for exact matches of short stretches (6-8 contiguous amino acids) of sequence that could serve as potential linear IgE binding sites indicated the existence of no matches with 8-amino acid stretches, one 7-amino acid match (with Asp n 14 & Asp n ?), and five 6-amino acid matches (with Act c a, Cuc m 1, Phl p 5 & Lol p 5, Hev b 5, and Cla h 6). According to Fiers et al., [38] the use of short contiguous sequences may lead to a high false positive rate, as high as 75% for 6 amino acids and Codex[37] recommends validation of the techniques before any conclusions can be drawn.

Summarizing those hits, most of these short matches are not with food allergens, except Act c a (not described by IUIS) and Cuc m 1 (described by IUIS) (Allergen Nomenclature,

International Union of Immunological Societies (www.allergen.org). Although cross-reactivity cannot be excluded, most inhalation or skin allergens are not active via the oral route.

Of the matching allergens, Hev b 5 has undergone a full analysis of IgE epitopes. The 6-amino acid stretch within TG that matches with Hev b 5 is not among the described epitopes of Hev b 5. Further, two additional amino acid stretches identified (matching Asp n 14 & Asp n ?, and Phl p 5 & Lol p 5) significantly overlap with the one matching with Hev b 5, and hence, these stretches can also be eliminated as potential epitopes.

To further explore the allergenic potential of the short contiguous stretches of amino acids matching known allergen sequences, protein hydrophylicity analysis was undertaken with ExPaSy ProtScale software (http://www.expasy.org/cgi-bin/protscale.pl) using the Hopp & Woods hydrophylicity algorithm[40]. This analysis indicates none of the six short amino acid stretches in the TG sequence to be hydrophilic; if not hydrophilic, then these amino acids stretches unlikely represent epitopes exposed to the surface of a folded protein.

In conclusion, using a weight-of-evidence approach, TG is unlikely to pose a risk of food allergenicity as 1) no hits with known allergens were found using the 35% match over 80-amino acids criterion, and 2) most matches with short contiguous sequence stretches of known allergens were of 6 amino acids only, and could be eliminated as likely epitopes as they don't match the known epitopes of the relevant allergen or due to the physicochemical properties (lack of strong hydrophylicity) of the matching regions.

Given the above sequence and structure analysis, the potential for respiratory sensitization to TG appears equally low. Nevertheless, as for all enzyme products, the MSDS for the TG 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

Transglucosidase from *A. niger* has been determined to be Generally Recognized as Safe (GRAS) by scientific procedures by Genencor and other enzyme companies. TG from *A. niger* is described in GRSN 246[35] as being used to form the α-1 -6 linkages comprising the isomalto-oligosaccharide mixtures to be used in a variety of foods; the agency response letter and the GRAS Notice are available on the FDA's GRAS website[29]. TG from *A. niger* has also been evaluated by many other regulatory bodies around the world, including those in Canada, Australia/New Zealand, and China, and determined to be safe for use in food processing.

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.

As mentioned in Section 7.1.2, Genencor has conducted two safety studies on the *T. reesei* organism itself, one on a recombinant strain derived from RL-P37, modified to overexpress an endoglucanase enzyme, and another on strain A83, also derived from RL-P37 through mutation and selection (see Appendix 3 for summary of the safety studies). We have also conducted many safety studies on *T. reesei* derived enzymes (see Appendix 3). Over the years, Genencor scientists have used several methods to modify RL-P37, including using different selectable markers with PEG-mediated or *Agrobacterium*-mediated gene transfer. As confirmed by the results of the safety testing of both the production organisms and the products, *T. reesei* RL-P37 is a safe production host, and the enzyme preparations resulting from it are safe for use in food and feed.

Based on the publicly available scientific information and confirmed by the results of the Genencor safety studies conducted on *T. reesei* RL-P37 derivative strains and RL-P37 derived products, and the GRAS status of *A. niger* TG, Genencor, using the safe strain lineage concept of Pariza and Johnson (See Appendix 4), determined that no additional safety studies on TG are warranted.

7. 4. Overall Safety Assessment and Human Exposure

7.4.1 Human Exposure Assessment

Transglucosidase (TG) is used as a processing aid in:

- Production of IMO (isomalto-oligosaccharide) syrup from starch from a variety of sources (e.g. corn, wheat, rice, etc.), and
- Production of potable alcohol from molasses.

While we expect no TG to remain in the IMO or potable alcohol, the following calculations are based on the assumption that 100% of the enzyme remains in the processed food.

7.4.1.1 Isomalto oligosaccharide (IMO) syrup from dry starch

The application rate of TG product is 0.5 to 1.5 kg per metric tonne of dry starch. Using a worst case scenario approach, the dose of 1.5 kg of TG product is used:

1.5 kg TG product in 1000 kg dry starch (DS).

Since the total protein concentration in the product is 5% (2000 – 2500 TGU/g), then the application of TG (as total protein) is:

1.5 kg TG product in 1000 kg DS x 5% =
75 g TG total protein in 1000 kg DS.

Assuming that the yield of IMO syrup from dry starch is 100% (worst case), then:
75 g TG in 1000 L of IMO syrup, or
0.075 g TG in 1 L of IMO syrup, or
75 mg TG in 1 L of IMO syrup, or
0.075 mg TG in 1 ml of IMO syrup.

7.4.1.2 Potable alcohol from molasses

The application rate of TG product in the production of ethanol from molasses is 6 to 20 ppm added during fermentation. Using a worst case scenario approach, the dose of 20 ppm is used:

20 ppm = 20 mg per kg of molasses, or
20 gram per 1000 kg (1 metric tonne) of molasses.

Since the total protein concentration in the product is 5% (2000 – 2500 TGU/ml), then the application of TG (as total protein) is:

20 g TG product in 1000 kg molasses x 5% =
1 g TG total protein in 1000 kg of molasses.

Since the yield of ethanol from molasses is 200 to 250 liters (95%) per metric tonne, the concentration of TG found in alcohol is:

1 g TG per 1000 kg of molasses =
1 g TG per 250 liters alcohol (95%) =
(worst case – assuming that all TG is transferred from molasses)
4 mg TG per liter alcohol (95%) =
0.004 mg TG per ml alcohol (95%).

7.4.2 Estimated Human Intake

While we expect no TG to remain in the IMO or potable alcohol, the following calculations are based on the assumption that 100% of the enzyme remains in the processed food.

7.4.2.1 Isomalto oligosaccharide syrup

In the FDA Response letter to GRAS Notice No. GRN 000246 (Feb 10, 2009)[35], an intake of IMO syrup at approximately 30 grams/person/day is used as the estimated intake for risk assessment. This estimate is based on the assumption of two servings of foods containing the highest use level identified (15 gram/serving). Since the specific gravity of IMO syrup is 1.46, the corresponding volume of IMO syrup consumed daily is therefore 20.55 ml.

Estimated maximum intake of TG from IMO syrup:

0.075 mg TG per ml of IMO syrup x 20.55 ml =
1.55 mg TG/person/day =
0.03 mg TG/kg bw/day.
(using 60 kg body weight as default).

7.4.2.2 Potable alcohol

Based on the USDA, the annual human intake of alcoholic beverages is 25 gallons (including beer, ale, wine, and liqueurs) or 94.5 liters. The daily consumption of alcoholic beverages is:

94.5 liters : 365 days = 0.26 liter/day or 260 ml/day.

To represent a worst case scenario, it is assumed that:
a. 100% of alcoholic beverages consumed daily are derived from molasses treated with TG product
b. 100% of TG remains in potable alcohol, although none is expected
c. The average concentration of potable alcohol in the alcoholic beverages is 20% (40° proof),

Daily consumption of 95% alcohol from alcoholic beverage:

$$\frac{260 \text{ ml/kg bw/day} \times 20}{95} = 55 \text{ ml /day.}$$

Estimated maximum daily intake of TG product:

0.004 mg TG per ml alcohol x 55 ml/day = 0.22 mg/person/day
0.004 mg TG/kg bw/day.
(using 60 kg body weight as default)

7.4.2.3 Cumulative exposure

Under the worst case scenario, the cumulative daily exposure to TG (expressed as total protein) from intake of IMO syrup and potable alcohol is:

0.03 mg/kg bw/day + 0.004 mg/kg bw/day = 0.034 mg/kg bw/day.

7.4.3 Identification of the NOAEL

Genencor has conducted a 90-day oral (gavage) study in rats (Scantox No. 60623) with a protease produced from a genetically modified strain of *T. reesei* which is derived from the same production host and strain lineage as used for TG. In that study, a NOAEL was established at 31.25 mg total protein/kg bw/day (equivalent to 35.81 mg TOS/kg bw/day).

The study was conducted in compliance with both the FDA Good Laboratory Practice Regulations and the OECD Good Laboratory Practice and was designed based on OECD guideline No. 408. Since human exposure to TG is through oral ingestion, selection of this NOAEL is thus appropriate.

7.5 Conclusion

Determination of safety factor

Based on the results from the 90-day oral (gavage) feeding study cited above

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

$$\text{Margin of safety} = \frac{31.25 \text{ mg/kg bw/day}}{0.034 \text{ mg/kg bw/day}} = 919$$

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, acid fungal protease, chymosin and α-amylase enzyme preparations, are well recognized by qualified experts as being safe for their intended uses. Published literature, government laws and regulations, reviews by expert panels such as FAO/WHO JECFA (1992)[33], as well as Genencor's own unpublished safety studies, support such a conclusion.

Trichoderma 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. In addition, the *T. reesei* lineage used by Genencor has been demonstrated to be safe.

Transglucosidase produced by *A. niger*, the source of the gene for this product, is GRAS. Based on TOS and extrapolation of the NOAEL derived from a similar enzyme within the same *T. reesei* safe strain lineage, this TG has a margin of safety of >900 for its anticipated uses in IMO and potable ethanol manufacture.

Based on the publicly available scientific data from the literature and additional supporting data generated by Genencor, the company has concluded that transglucosidase from *Trichoderma reesei* strain TG #626, GICC 03289 is safe and suitable for use in IMO manufacture and production of potable ethanol from molasses and is Generally Recognized as Safe (GRAS). In addition, the safety determination, including construction of the production organism, the production process and materials, and safety of the product, were reviewed by Dr. Michael Pariza, and he concurred with the company's conclusion that the product is GRAS (see Appendix 5).

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Appendix 1 – The amino acid sequence of the transglucosidase

Appendix 2 - The Manufacturing process

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

Appendix 4 - The Pariza/Johnson Decision Tree

Appendix 5 – GRAS Panel Report

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GENENCOR
A Danisco Division

December 15, 2009

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Ms. Moraine Ramos

Office of Food Additive Safety (HFS-255)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
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Dear Ms. Ramos:

Per your request by telephone on December 14, 2009 for corrections to Danisco's GRAS Notification for transglucosidase enzyme preparation produced by *Trichoderma reesei* expressing the gene encoding transglucosidase from *Aspergillus niger*, please find enclosed three copies of the corrected document.

As the inserted text resulted in some of the pages to get re-arranged, we elected to resubmit the entire main document with exception of the appendices, for which the page number remained unchanged from the original submission. Also enclosed is a new Appendix 6.

If you have questions or require additional information, please contact me at 650-846-5861 or fax at 650-845-6505.

Sincerely,

(b) (6)

Vincent J. Sewalt
Director of Regulatory Affairs

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Encl.

GENENCOR

Transglucosidase Enzyme Preparation Derived from

Trichoderma reesei

Expressing the Transglucosidase Gene

from

***Aspergillus niger* Is Generally Recognized As Safe**

For Use in Food Processing

Notification Submitted by Genencor, a Danisco Division

December 4, 2009

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1. GENERAL INTRODUCTION

The transglucosidase (TG) enzyme preparation under consideration is produced by submerged fermentation of *Trichoderma reesei* carrying the gene encoding the transglucosidase enzyme from *Aspergillus niger*. This commercial TG product will replace the current *A. niger* derived TG.

Use of the enzyme product is intended to convert the malto-oligosaccharides in starch from grains to isomalto-oligosaccharides (IMO) and as one of the enzymes in an enzyme cocktail used to produce potable alcohol from molasses. In these applications, the *Trichoderma* transglucosidase will primarily be replacing transglucosidase from the *A. niger* commercial sources. In this application, transglucosidase will be used as a processing aid where the enzyme is not present in the final food.

The systematic name of the principle enzyme activity is 1,4-α-D-glucan:1,4-α-D-glucan (D-glucose) 6-α-D-glucosyltransferase. Other names used are Oligoglucan-branching glycosyltransferase; 1,4-α-D-glucan 6-α-D-glucosyltransferase; T-enzyme; D-glucosyltransferase, and transglucosidase.

The enzyme catalyses both hydrolytic and transfer reactions on incubation with α-D-gluco-oligosaccharides. Transfer occurs most frequently to HO-6, producing isomaltose from D-glucose, and panose from maltose. Transglucosidase can also transfer to the HO-2 or HO-3 of D-glucose to form kojibiose or nigerose, or back to HO-4 to form maltose. The action on maltose produces equimolar concentration of panose and glucose.

The EC number of the enzyme is 2.4.1.24, and the CAS number is 9030-12-0.

Pursuant to the regulatory and scientific procedures established in proposed 21 C.F.R. 170.36, Danisco has determined that its TG enzyme preparation from a modified strain of *T. reesei* expressing the transglucosidase enzyme from *Aspergillus niger* is a Generally Recognized As Safe ("GRAS") substance for the intended food application and is, therefore, exempt from the requirement for premarket approval. The information provided in the following sections is the basis of our determination of GRAS status of this TG 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 for this TG, *T. reesei,* 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.[3-8] 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.

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 transglucosidase enzyme preparation produced by *Trichoderma reesei* expressing the gene encoding transglucosidase from *Aspergillus niger* 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

Transglucosidase enzyme preparation from *Trichoderma reesei* expressing the gene encoding the transglucosidase enzyme from *Aspergillus niger*

1.4 Applicable Conditions of Use

Transglucosidase is used as a processing aid in the production of IMO syrups from starch and in the production of potable ethanol from molasses. To our knowledge, use of transglucosidase is the only method of producing IMOs from starch.

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. 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.

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The production organism is a strain of *Trichoderma reesei*, which has been genetically modified by deletion of several cellulase genes and to express a gene for the production and secretion of transglucosidase isolated from *A. niger* (see Section 2.2). *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]. It contains the transglucosidase gene under the regulation of a native *T. reesei cbhI* (cellobiohydrolase 1) gene. The *A. nidulans amdS* gene was used as a selectable marker. The inserted DNA was integrated into the recipient chromosome.

2.2 Host Microorganism

The host organism *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 *Trichoderma 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 Transglucosidase Expression Vector

For construction of the vector, the DNA encoding the *A. niger* mature secreted transglucosidase protein was fused to the *T. reesei* CBHI signal peptide. This open reading frame is flanked by the promoter and terminator sequences of the *T. reesei cbh1* gene. The vector also contains the *Aspergillus nidulans amdS* gene, encoding acetamidase, as a selectable marker for transformation of *T. reesei.*

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 that only the intended genetic modifications to the *T. reesei* strain had been made.

2.4 Stability of the Introduced Genetic Sequences

The production strain proved to be 100% stable after at least 60 generations of fermentation, judged by transglucosidase production.

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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 1CFU/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]:	1,4-α-glucan-6-α-glucosyltransferase
IUBMB Number:	2.4.1.24
CAS Number:	9030-12-0
Reaction catalyzed:	Transglucosidase transfers an α-D-glucosyl residue in a 1,4-α-D-glucan to the primary hydroxyl group of glucose, free or combined in a 1,4-α-D-glucan.
Other Names:	Oligoglucan-branching glycosyltransferase; 1,4-α-D-glucan 6-α-D-glucosyltransferase; T-enzyme; D-glucosyltransferase[12].
Systematic Name:	1,4-α-D-glucan:1,4-α-D-glucan (D-glucose) 6-α-D-glucosyltransferase[12].

Technical Effect:
Transglucosidase catalyses both hydrolytic and transfer reactions on incubation with α-D-gluco-oligosaccharides. Transfer occurs most frequently to HO-6, producing isomaltose from D-glucose, and panose from maltose. Transglucosidase can also transfer to the HO-2 or HO-3 of D-glucose to form kojibiose or nigerose, or back to HO-4 to form maltose. The action on maltose produces equimolar concentration of panose and glucose.

As the result of transglucosidase reactions, the malto-oligosaccharides are converted to isomalto-oligosaccharides containing high proportions of glucosyl residues linked by an α-D-1,6 linkage from the non-reducing end. In molasses, non-fermentable sugars including raffinose and stachyose are converted to sucrose, galactose, glucose and fructose, which can then be fermented into alcohol.

3.2 Amino Acid Sequence

The amino acid sequence of the TG enzyme and nucleotide sequence of the gene encoding it are freely available from public databases such as GenBank (Accession numbers BAA23616, D45356). The sequence corresponding to the mature transglucosidase (between the positions 1251 and 1252 in the GenBank sequence D45356,[33,34] was used for this construction.

The amino acid sequence of the TG enzyme is shown in Appendix 1.

4. MANUFACTURING PROCESS

This section describes the manufacturing process for this TG enzyme which follows standard industry practice.[23-25] 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 TG concentrate are standard ingredients used in the enzyme industry.[23-25] All the raw materials conform to the specifications of the Food Chemicals Codex, 6th edition, 2008 ("FCC"),[14] 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 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 and flocculants used in the fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 24, 1998. The maximum use level of these antifoam in the production process is $\leq$0.15% and of the flocculants <1.5%.

4.2 Fermentation Process

The TG enzyme is manufactured by submerged straight-batch or fed-batch pure culture fermentation of the genetically modified strain of *T. reesei* described in Section 2. All equipment is carefully designed, constructed, operated, cleaned and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are conducted periodically to ensure absence of foreign microorganisms and confirm production strain identity.

4.2.1 Production organism

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

4.2.2 Criteria for the rejection of fermentation batches

Growth characteristics during fermentation are observed microscopically. Samples are taken from each fermentation stage (inoculum, seed-, and main fermentor) before inoculation, at regular intervals during growth and before harvest or transfer. These samples are tested for microbiological contamination by plating on a nutrient medium.

A fermentation batch is declared as 'contaminated' if colony forming units (CFU) of bacteria or fungi other than the production strain are present at levels $>10^3$CFU/ml.

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. Polish filtration – for removal of residual production strain organisms and as a general precaution against microbial degradation
3. Concentration – ultrafiltration
4. Formulation.

4.3.2 Formulation and standardization process

The ultrafiltered concentrate is stabilized with 3 % sodium chloride and 50% glycerol.

4.3.3 Quality control of finished product

The final TG 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")[15] in 2006 and the FCC[14]. These specifications are set forth in Section 5.

5. COMPOSITION AND SPECIFICATIONS

5.1 Quantitative Composition

The liquid concentrate is stabilized with 3 % sodium chloride and 50% glycerol and tested to demonstrate that it meets the specifications in section 5.2.

The formulation of the enzyme preparation is as follows:

- 3 % enzyme protein
- 3 % sodium chloride
- 50% glycerol
- 44% water.

5.2 Specifications

Transglucosidase meets the purity specifications for enzyme preparations set forth in Food Chemicals Codex[14]. In addition, it also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications [15].

6. APPLICATION

6.1 Mode of Action

Transglucosidase catalyses both hydrolytic and transfer reactions on incubation with α-D-gluco-oligosaccharides. Transfer occurs most frequently to HO-6, producing isomaltose from D-glucose, and panose from maltose. Transglucosidase can also transfer to the HO-2 or HO-3 of D-glucose to form kojibiose or nigerose, or back to HO-4 to form maltose. The action on maltose produces equimolar concentrations of panose and glucose.

As the result of transglucosidase reactions, the malto-oligosaccharides are converted to isomalto-oligosaccharides containing high proportions of glucosyl residues linked by an α-D-1,6 linkage from the non-reducing end. In molasses, non-fermentable sugars including raffinose and stachyose are converted to sucrose, galactose, glucose and fructose, which can then be fermented into alcohol.

6.2 Use Levels

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Transglucosidase is used as a processing aid in the production of IMO syrups from starch and in the production of potable ethanol from molasses. To our knowledge, use of transglucosidase is the only method of producing IMOs from starch.

IMO
The dose of TG product for IMO production is 0.5 to 1.5 kg per ton of DS. After about two days' reaction, the enzyme will be deactivated at about 80°C. Followed by carbon treatment for decoloration, then ion exchange, so there should be no enzyme left in the end product similar to other saccharifying enzymes for glucose or maltose production.

Potable Ethanol
The enzyme product dose is 6-20 ppm, added during fermentation. After about 10 to 24 hours fermentation, the broth will go through distillation, where all the enzyme will be deactivated.

6.3 Enzyme Residues in the Final Foods

As discussed above, the enzyme will be deactivated in both applications and will not be present in the final food products.

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[1] 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 that *T. reesei* be placed into synonymy with *T. longibrachiatum*[13]. Later however, evidence emerged indicating that the two species are not identical[9]. 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[17]. Their work was confirmed by another group that found evidence of peptaibol production in two other *T. reesei* strains[18]. 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 the FCC.

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.[19], Blumenthal[20] and Olemska-Beer et al.[21]. 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 various strains have been approved to produce commercial enzyme products internationally, for example, in Canada (Food and Drugs Act Division 16, Table V, Food Additives That May Be Used As Enzymes), the United States (FDA 1999), France, Australia/New Zealand, China (MOH 1996), and Japan.

The production organism of the transglucosidase enzyme preparation, the subject of this submission, is *T. reesei* strain TG #626, GICC 03289. It is derived by recombinant DNA methods from strain RL-P37. The purpose of this genetic modification is to express the transglucosidase from *A. niger* in *T. reesei*. RL-P37, a commercial production strain, is derived, as a result of 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 strain has been determined to be non-

pathogenic and non-toxicogenic through an acute intraperitoneal study in rats. All of the food/feed grade products produced by this lineage were determined to be safe for their intended uses and are the subject of numerous GRAS determinations. A GRAS Notice was filed for one of the products from this strain lineage and FDA issued a no questions letter (see GRAS Notice 230[42]).

From the information reviewed, it is concluded that the organism *T. reesei* provides no specific risks to human health and is safe to use as the production organism of transglucosidase.

It is concluded that the strain is non-pathogenic and non-toxigenic.

7.1.2 Safety of the donor source

Aspergillus niger has a long history of safe use in the production of industrial enzymes and chemicals of both food grade and technical grade. It is one of the most important producers of industrial neutral proteases[22] and glucoamylases[23]. The species is listed as a production/donor organism for a series of food-grade carbohydrases, oxidoreductases, lipases, glucanotransferase, and proteases in the recent Pariza and Johnson paper[1]. Several *A. niger*-derived substances used in the food industry have acquired GRAS status for the proposed applications:

- Carbohydrase and cellulase enzyme preparation (21 CFR §173.120)[26];
- Citric acid (21 CFR §173.280)[27];
- Carbohydrase enzyme preparation, catalase enzyme preparation, glucose oxidase enzyme preparation, pectinase enzyme preparation, and protease enzyme preparation (GRSN 000089)[28]; and
- Lactase (GRSN 0000132); Lipase (GRSN 0000111), Asparaginase (GRSN 000214), Phosplipase A2 (GRSN 0000183)[29].

The safety of *A. niger* was summarized by Schuster *et al*[30] and was also discussed by Olempska-beer *et al*[21].

In 1997, *A. niger* became one of the ten microbial species/strains that were eligible for exemption under 40 CFR Part 725 as recipient microorganisms under the TSCA biotechnology regulations[31], based on EPA's risk assessments.

The donor organism for the transglucosidase gene used in construction of the new microorganism *T. reesei* TG #626, GICC 03289 was *Aspergillus niger* AGME 9, also known as SE4, and described in US patent 5,783,414. *A. niger* AGME9 is the source of Genencor's current transglucosidase product. The enzyme from *A. niger* is one of the well known forms of transglucosidases[32]. The amino acid sequence of the enzyme and nucleotide sequence of the gene encoding it are freely available from public databases such as GenBank (Accession numbers BAA23616, D45356). The sequence corresponding to the mature transglucosidase (between the positions 1251 and 1252 in the GenBank sequence D45356,[33,34] was used for this construction.

The *A. niger* transglucosidase is GRAS as previously determined by Genencor and concurred with by Dr. Michael Pariza. Additionally, it is described as the enzyme used to produce isomalto-oligosaccharides in GRAS Notice 000246 and the corresponding FDA no-questions letter[35].

7.2 Safety of the Manufacturing Process

The manufacturing process for the production of transglucosidase will be conducted in a manner similar to other food and feed production processes. It consists of a pure-culture fermentation process, cell separation, concentration and formulation, resulting in a liquid transglucosidase 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, 6th Edition[14] and the general specifications for enzyme preparations used in food processing proposed by WHO/JECFA[15].

7.3 Safety of Transglucosidase

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. Transglucosidases have been used in food processes to generate IMOs for several years and have generated no known safety concerns.

In 1998 the Association of Manufacturers of Fermentation Enzyme Products (AMFEP) Working Group on Consumer Allergy Risk from Enzyme Residues in Food reported on an in-depth analysis of the allergenicity of enzyme products. They concluded that there are no scientific indications that small amounts of enzymes in bread and other foods can sensitize or induce allergy reactions in consumers, and that enzyme residues in bread and other foods do not represent any unacceptable risk to consumers. Further, in a recent investigation of possible oral allergenicity of 19 commercial enzymes used in the food industry, there were no findings of clinical relevance even in individuals with inhalation allergies to the same enzymes, and the authors concluded "that ingestion of food enzymes in general is not considered to be a concern with regard to food allergy[36]."

Despite this lack of general concern, the potential that transglucosidase could be a food allergen was assessed by comparison with sequences of known allergens and is summarized here. FASTA alignments of the *A. niger* TG sequence with known allergens using both the SDAP database (http://fermi.utmb.edu/SDAP/sdap_who.html)containing 887 allergens (listed in http://fermi.utmb.edu/cgi-bin/SDAP/sdap_01) or the combined SwissProt and WHO-IUIS databases in Allermatch (www.allermatch.org) containing 792 allergens reveals no significant (E<0.01) homology with any known allergens.

Following the Codex (2003)[37] guidelines, homology searches were performed against the combined Allermatch database as well as the SDAP database, using a sliding window of 80-amino acids and greater than 35% identity to indicate a match, which revealed no matches of *A. niger* TG with any known allergens. This Codex recommended search is most authoritative as it reduces the likelihood of false negatives or false positives[38,39].

A secondary, more controversial search for exact matches of short stretches (6-8 contiguous amino acids) of sequence that could serve as potential linear IgE binding sites indicated the existence of no matches with 8-amino acid stretches, one 7-amino acid match (with Asp n 14 & Asp n ?), and five 6-amino acid matches (with Act c a, Cuc m 1, Phl p 5 & Lol p 5, Hev b 5, and Cla h 6). According to Fiers et al., [38] the use of short contiguous sequences may lead to a high false positive rate, as high as 75% for 6 amino acids and Codex[37] recommends validation of the techniques before any conclusions can be drawn.

Summarizing those hits, most of these short matches are not with food allergens, except Act c a (not described by IUIS) and Cuc m 1 (described by IUIS) (Allergen Nomenclature, International Union of Immunological Societies (www.allergen.org). Although cross-reactivity cannot be excluded, most inhalation or skin allergens are not active via the oral route.

Of the matching allergens, Hev b 5 has undergone a full analysis of IgE epitopes. The 6-amino acid stretch within TG that matches with Hev b 5 is not among the described epitopes of Hev b 5. Further, two additional amino acid stretches identified (matching Asp n 14 & Asp n ?, and Phl p 5 & Lol p 5) significantly overlap with the one matching with Hev b 5, and hence, these stretches can also be eliminated as potential epitopes.

To further explore the allergenic potential of the short contiguous stretches of amino acids matching known allergen sequences, protein hydrophylicity analysis was undertaken with ExPaSy ProtScale software (http://www.expasy.org/cgi-bin/protscale.pl) using the Hopp & Woods hydrophylicity algorithm[40]. This analysis indicates none of the six short amino acid stretches in the TG sequence to be hydrophilic; if not hydrophilic, then these amino acids stretches unlikely represent epitopes exposed to the surface of a folded protein.

In conclusion, using a weight-of-evidence approach, TG is unlikely to pose a risk of food allergenicity as 1) no hits with known allergens were found using the 35% match over 80-amino acids criterion, and 2) most matches with short contiguous sequence stretches of known allergens were of 6 amino acids only, and could be eliminated as likely epitopes as they don't match the known epitopes of the relevant allergen or due to the physicochemical properties (lack of strong hydrophylicity) of the matching regions.

Given the above sequence and structure analysis, the potential for respiratory sensitization to TG appears equally low. Nevertheless, as for all enzyme products, the MSDS for the TG 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

Transglucosidase from *A. niger* has been determined to be Generally Recognized as Safe (GRAS) by scientific procedures by Genencor and other enzyme companies. TG from *A. niger* is described in GRSN 246[35] as being used to form the α-1 -6 linkages comprising the isomalto-oligosaccharide mixtures to be used in a variety of foods; the agency response letter and the GRAS Notice are available on the FDA's GRAS website[29]. TG from *A. niger* has also been evaluated by many other regulatory bodies around the world, including those in Canada, Australia/New Zealand, and China, and determined to be safe for use in food processing.

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.

As mentioned in Section 7.1.2, Genencor has conducted two safety studies on the *T. reesei* organism itself, one on a recombinant strain derived from RL-P37, modified to overexpress an endoglucanase enzyme, and another on strain A83, also derived from RL-P37 through mutation and selection (see Appendix 3 for summary of the safety studies). We have also conducted many safety studies on *T. reesei* derived enzymes (see Appendix 3). Over the years, Genencor scientists have used several methods to modify RL-P37, including using different selectable markers with PEG-mediated or *Agrobacterium*-mediated gene transfer. As confirmed by the results of the safety testing of both the production organisms and the products, *T. reesei* RL-P37 is a safe production host, and the enzyme preparations resulting from it are safe for use in food and feed.

Based on the publicly available scientific information and confirmed by the results of the Genencor safety studies conducted on *T. reesei* RL-P37 derivative strains and RL-P37 derived products, and the GRAS status of *A. niger* TG, Genencor, using the safe strain lineage concept of Pariza and Johnson (See Appendix 4), determined that no additional safety studies on TG are warranted.

7. 4. Overall Safety Assessment and Human Exposure

7.4.1 Human Exposure Assessment

Transglucosidase (TG) is used as a processing aid in:

- Production of IMO (isomalto-oligosaccharide) syrup from starch from a variety of sources (e.g. corn, wheat, rice, etc.), and
- Production of potable alcohol from molasses.

While we expect no TG to remain in the IMO or potable alcohol, the following calculations are based on the assumption that 100% of the enzyme remains in the processed food.

7.4.1.1 Isomalto oligosaccharide (IMO) syrup from dry starch

The application rate of TG product is 0.5 to 1.5 kg per metric tonne of dry starch. Using a worst case scenario approach, the dose of 1.5 kg of TG product is used:

1.5 kg TG product in 1000 kg dry starch (DS).

Since the total protein concentration in the product is 5% (2000 – 2500 TGU/g), then the application of TG (as total protein) is:

1.5 kg TG product in 1000 kg DS x 5% =
75 g TG total protein in 1000 kg DS.

Assuming that the yield of IMO syrup from dry starch is 100% (worst case), then:
75 g TG in 1000 L of IMO syrup, or
0.075 g TG in 1 L of IMO syrup, or
75 mg TG in 1 L of IMO syrup, or
0.075 mg TG in 1 ml of IMO syrup.

7.4.1.2 Potable alcohol from molasses

The application rate of TG product in the production of ethanol from molasses is 6 to 20 ppm added during fermentation. Using a worst case scenario approach, the dose of 20 ppm is used:

20 ppm = 20 mg per kg of molasses, or
20 gram per 1000 kg (1 metric tonne) of molasses.

Since the total protein concentration in the product is 5% (2000 – 2500 TGU/ml), then the application of TG (as total protein) is:

20 g TG product in 1000 kg molasses x 5% =
1 g TG total protein in 1000 kg of molasses.

Since the yield of ethanol from molasses is 200 to 250 liters (95%) per metric tonne, the concentration of TG found in alcohol is:

1 g TG per 1000 kg of molasses =
1 g TG per 250 liters alcohol (95%) =
(worst case – assuming that all TG is transferred from molasses)
4 mg TG per liter alcohol (95%) =

0.004 mg TG per ml alcohol (95%).

7.4.2 Estimated Human Intake

While we expect no TG to remain in the IMO or potable alcohol, the following calculations are based on the assumption that 100% of the enzyme remains in the processed food.

7.4.2.1 Isomalto oligosaccharide syrup

In the FDA Response letter to GRAS Notice No. GRN 000246 (Feb 10, 2009)[35], an intake of IMO syrup at approximately 30 grams/person/day is used as the estimated intake for risk assessment. This estimate is based on the assumption of two servings of foods containing the highest use level identified (15 gram/serving). Since the specific gravity of IMO syrup is 1.46, the corresponding volume of IMO syrup consumed daily is therefore 20.55 ml.

Estimated maximum intake of TG from IMO syrup:

0.075 mg TG per ml of IMO syrup x 20.55 ml =
1.55 mg TG/person/day =
0.03 mg TG/kg bw/day.
(using 60 kg body weight as default).

7.4.2.2 Potable alcohol

Based on the USDA, the annual human intake of alcoholic beverages is 25 gallons (including beer, ale, wine, and liqueurs) or 94.5 liters. The daily consumption of alcoholic beverages is:

94.5 liters : 365 days = 0.26 liter/day or 260 ml/day.

To represent a worst case scenario, it is assumed that:
a. 100% of alcoholic beverages consumed daily are derived from molasses treated with TG product
b. 100% of TG remains in potable alcohol, although none is expected
c. The average concentration of potable alcohol in the alcoholic beverages is 20% (40° proof),

Daily consumption of 95% alcohol from alcoholic beverage:

$$\frac{260 \text{ ml/kg bw/day} \times 20}{95} = 55 \text{ ml /day.}$$

Estimated maximum daily intake of TG product:

0.004 mg TG per ml alcohol x 55 ml/day = 0.22 mg/person/day

= 0.004 mg TG/kg bw/day.
(using 60 kg body weight as default)

7.4.2.3 Cumulative exposure

Under the worst case scenario, the cumulative daily exposure to TG (expressed as total protein) from intake of IMO syrup and potable alcohol is:

0.03 mg/kg bw/day + 0.004 mg/kg bw/day = 0.034 mg/kg bw/day.

As TG contains 106.4 mg total protein/ml or 12.51% TOS/ml, the cumulative daily exposure, expressed as TOS, is 0.040 mg/kg bw/day.

7.4.3 Identification of the NOAEL

Genencor has conducted a 90-day oral (gavage) study in rats (Scantox No. 60623) with a protease produced from a genetically modified strain of *T. reesei* which is derived from the same production host and strain lineage as used for TG. The Certificate of Analysis for the tox lot used in that study is enclosed (see Appendix 6. In that study, a NOAEL was established at 31.25 mg total protein/kg bw/day (equivalent to 35.81 mg TOS/kg bw/day). The study was conducted in compliance with both the FDA Good Laboratory Practice Regulations and the OECD Good Laboratory Practice and was designed based on OECD guideline No. 408. Since human exposure to TG is through oral ingestion, selection of this NOAEL is thus appropriate.

7.5 Conclusion

Determination of safety factor

Based on the results from the 90-day oral (gavage) feeding study cited above

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

$$\text{Margin of safety} = \frac{35.81 \text{ mg TOS/kg bw/day}}{0.040 \text{ mg TOS/kg bw/day}} = 895$$

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, acid fungal protease, chymosin and α-amylase enzyme preparations, are well recognized by qualified experts as being safe for their intended uses. Published literature, government laws and regulations, reviews by expert

panels such as FAO/WHO JECFA (1992)[33], as well as Genencor's own unpublished safety studies, support such a conclusion.

Trichoderma 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. In addition, the *T. reesei* lineage used by Genencor has been demonstrated to be safe.

Transglucosidase produced by *A. niger*, the source of the gene for this product, is GRAS. Based on TOS and extrapolation of the NOAEL derived from a similar enzyme within the same *T. reesei* safe strain lineage, this TG has a margin of safety of 895 for its anticipated uses in IMO and potable ethanol manufacture.

Based on the publicly available scientific data from the literature and additional supporting data generated by Genencor, the company has concluded that transglucosidase from *Trichoderma reesei* strain TG #626, GICC 03289 is safe and suitable for use in IMO manufacture and production of potable ethanol from molasses and is Generally Recognized as Safe (GRAS). In addition, the safety determination, including construction of the production organism, the production process and materials, and safety of the product, were reviewed by Dr. Michael Pariza, who concurred with the company's conclusion that the product is GRAS (see Appendix 5).

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10. LIST OF APPENDICES

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Appendix 2 - The Manufacturing process

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Appendix 6 – Composition of Tox Lot

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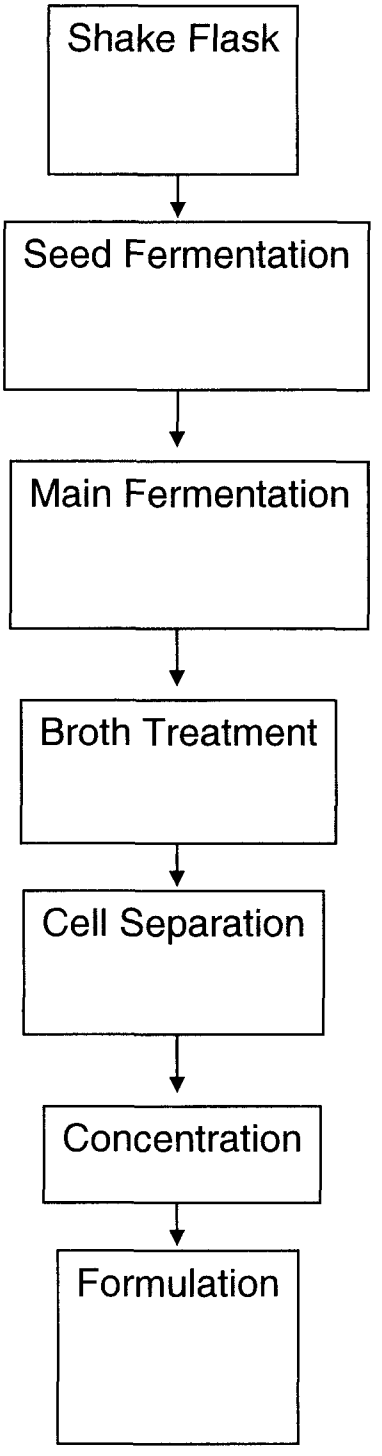
Appendix 1 – The amino acid sequence of transglucosidase

STTAPSQPQFTIPASADVGAQLIANIDDPQAADAQSVCPGYKASKVQHNSRGFTASLQLAGRPCNVYGTDVESLTLSV
EYQDSDRLNIQILPTHVDSTNASWYFLSENLVPRPKASLNASVSQSDLFVSWSNEPSFNFKVIRKATGDALFSTEGTV
LVYENQFIEFVTALPEEYNLYGLGEHITQFRLQRNANLTIYPSDDGTPIDQNLYGQHPFYLDTRYYKGDRQNGSYIPV
KSSEADASQDYISLSHGVFLRNSHGLEILLRSQKLIWRTLGGGIDLTFYSGPAPADVTRQYLTSTVGLPAMQQYNTLG
FHQCRWGYNNWSDLADVVANFEKFEIPLEYIWTDIDYMHGYRNFDNDQHRFSYSEGDEFLSKLHESGRYYVPIVDAAL
YIPNPENASDAYATYDRGAADDVFLKNPDGSLYIGAVWPGYTVFPDWHHPKAVDFWANELVIWSKKVAFDGVWYDMSE
VSSFCVGSCGTGNLTLNPAHPSFLLPGEPGDIIYDYPEAFNITNATEAASASAGASSQAAATATTTSTSVSYLRTTPT
PGVRNVEHPPYVINHDQEGHDLSVHAVSPNATHVDGVEEYDVHGLYGHQGLNATYQGLLEVWSHKRRPFIIGRSTFAG
SGKWAGHWGGDNYSKWWSMYYSISQALSFSLFGIPMFGADTCGFNGNSDEELCNRWMQLSAFFPFYRNHNELSTIPQE
PYRWASVIEATKSAMRIRYAILPYFYTLFDLAHTTGSTVMRALSWEFPNDPTLAAVETQFMVGPAIMVVPVLEPLVNT
VKGVFPGVGHGEVWYDWYTQAAVDAKPGVNTTISAPLGHIPVYVRGGNILPMQEPALTTREARQTPWALLAALGSNGT
ASGQLYLDDGESIYPNATLHVDFTASRSSLRSSAQGRWKERNPLANVTVLGVNKEPSAVTLNGQAVFPGSVTYNSTSQ
VLFVGGLQNLTKGGAWAENWVLEW

Appendix 2- Diagram of the Manufacturing Process



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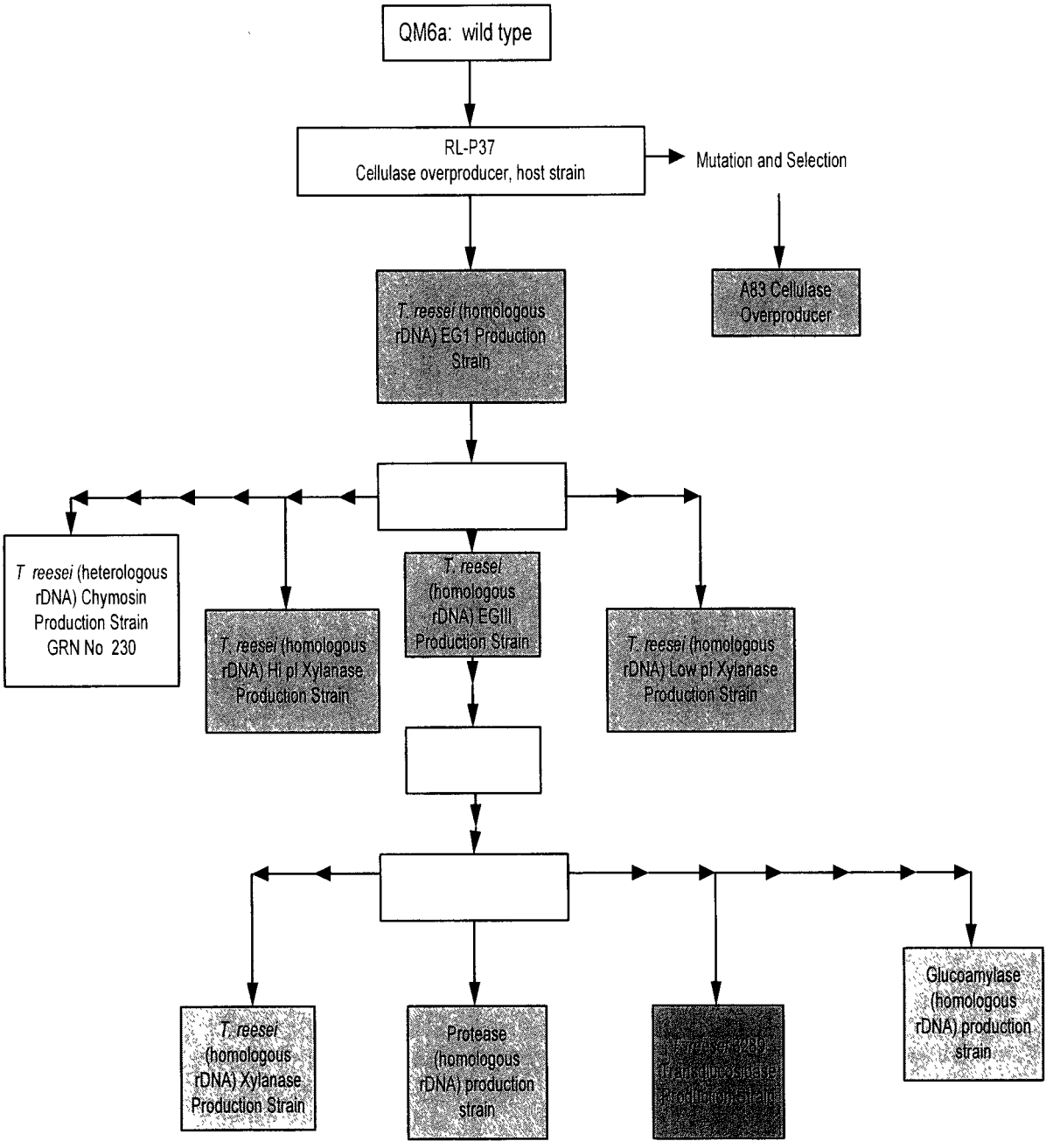
Appendix 3 – T. reesei Strain Lineage and Summary of Safety Studies

T. reesei Strain Lineage

Note: The number of steps between strains is generally more than is shown.



Please refer to the Toxicology Test Summaries chart on page 28 for explanation of the above.

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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

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)	Protease	91-day subchronic study, rats	No adverse effects detected
		Bacterial reverse mutation assay	Not mutagenic
		In vitro chromosome assay, human lymphocytes	Not clastogenic
		Acute dermal irritation in rabbits	Not a skin irritant
		Ocular irritation in rabbits	Not an eye irritant
		Acute oral toxicity in rats	Classified as non-hazardous (oral LD_{50} > 2000 mg/kg bw)

PRODUCTION ORGANISM	ENZYME PREPARATION	TOXICOLOGY TEST	RESULT
VIII. *T. reesei* (homologous rDNA)	Glucoamylase	Acute dermal irritation study in rabbits (sequential approach)	Not a skin 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. <u>28-day oral study in rats</u>

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 5.6×10^6 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. Protease from *T. reesei* (homologous rDNA)

A. 91-day subchronic oral study in rats

This study was conducted in accordance with OECD Guideline 408 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. Groups of 10 male and female SPF Sprague Dawley rats were administered orally by gavage with 0 (water for injection), 6.25, 12.5, or 31.25 mg total protein/kg body weight in a constant volume of 5 ml/kg body weight corresponding to 0, 7.16, 14.32 or 35.81mg TOS/kg bw/day, respectively (LAB Scantox, 2006).

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. 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).

B. Bacterial reverse mutation assay (Ames assay)

The test material, protease from *T. reesei* was tested in five strains of *Salmonella typhimurium* (TA98, TA100, TA 102, TA 1535 and TA1537) in the presence and absence of metabolic activation (Scantox, 2006). The assay was conducted in accordance with OECD Guideline 471 using the "treat and plate" procedure.

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. Triplicates plates were used at each test point. Five dose levels of protease were used in the main tests: 50, 160, 500, 1600 and 5000 µg/plate. The highest dose level tested (5000 µg/plate) is the maximum required by 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. In the main assays, 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, protease has not shown any evidence of mutagenic activity in the Ames assay and was classified as "not a mutagen".

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

The test material, acid fungal protease 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 (Scantox, 2006). The assay was conducted in accordance with OECD Guideline 473.

In a preliminary toxicity assay, HPBL cells were exposed to five concentrations of protease 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 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.

No biologically or statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with protease 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, protease concentrate did not induce chromosomal aberrations (both structural and numerical) in mammalian cells both in the presence and absence of metabolic activation up to and including the maximum recommended dosage level of 5000 μg/ml.

D. Acute oral toxicity in rats

The limit test was used with a starting dose of 2000 mg/kg bw based on total protein (Scantox, 2006). This study was conducted according to OECD Guideline No. 420 (Acure oral toxicity – Fixed dose procedure) and in compliance with the OECD Principles of Good Laboratory Practice (as revised in 1977).

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), protease can be classified as non-hazardous (oral LD_{50} > 2000 mg/kg bw).

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

A. Acute oral toxicity in rats – Fixed dose 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 (Scantox, 2008). 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 1996.3 mg total protein/kg bw or 2073 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). No mortality was recorded in this study. There were no overt signs of systemic toxicity throughout the 14-day observation period and at necropsy.

Under the conditions of this study, the oral LD_{50} was > 1996.3 mg total protein/kg bw (corresponding to 2073 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} > 2000 mg TOS/kg bw, TrGA is classified to GSH category 5 according to Annex 2 and Annex 3 in the OECD Guideline.

B. Bacterial Reverse Mutation Assay – Ames assay.

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 in the presence and absence of metabolic activation (Scantox, 2009). 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.

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.

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.

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

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

The objective of this assay was to investigate the potential of TrGA to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes) (Scantox, 2009). 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.

No biologically or statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with TrGA 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, PROTEASE concentrate did not induce chromosomal aberrations (both structural and numerical) in mammalian cells both in the presence and absence of metabolic activation up to and including the maximum recommended dosage level of 5000 μg/ml.

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

The objective of this study was to investigate the potential of TrGA to induce systemic toxicity after repeated daily oral administration to SPF Sprague Dawley rats (Taconic M&B, Denmark) of both sexes for 90 consecutive days (Scantox, 2009). Groups of 10 rats/sex each were gavaged daily with 0 (0.9% saline), 10, 50 or 150 mg total 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 and 0, 39.3, 196.5 or 589.5 GAU/kg bw/day, respectively.

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

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

All other microscopic findings were considered to be within the background incidence of findings reported in this age and strain of laboratory animals.

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 histopathologic examinations. Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established at the highest dose tested, 150 mg total protein/kg bw/day corresponding to 155.5 mg TOS/kg bw/day or 589.5 GAU/kg bw/day.

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Appendix 4 -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 transglucosidase 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, *A. niger* TG is GRAS and vectors have been used by Genencor for numerous constructions demonstrated to be safe. Go to 3c;

3c. **Is the test article free of transferable antibiotic resistance gene DNA?** Yes. Transformable DNA was not detected at or above the limit of 5 ng/ml in the enzyme preparation manufactured using this host and production process. 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. Yes, inserted DNA is well characterized, but complete characterization of the location of all insertions is not possible Go to 4.

4. **Is the introduced DNA randomly integrated into the chromosome?** In general, yes except as noted in 3e. 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. Given the targeted integration (see 4 above) there is no concern for pleiotropic effects. Go to 6.

6. **Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?** Yes. Yes, *T. reesei* safety as a production host and methods of modification are well documented and their safety has been confirmed through toxicology testing, **Accept.**

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Appendix 5 – GRAS Panel Report

GENENCOR

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

Michael W. Pariza, Member

September 4, 2009

Alice J. Caddow
Vice President, Regulatory
and Environmental Affairs
Danisco US Inc
925 Page Mill Road
Palo Alto, CA 94304

Dear Ms. Caddow,

I have reviewed the information you provided on a Danisco transglucosidase (TG) enzyme preparation that is produced by a non-pathogenic, non-toxigenic strain of *Trichoderma reesei* (formerly classified as *T. longibrachiatum*), strain TG #626, GICC 03289, which was genetically modified to over express the TG enzyme from *Aspergillus niger*. The intended uses of this TG product are to convert the malto-oligosaccharides in starch from grains to isomalto-oligosaccharides, and as one of the enzymes in an enzyme cocktail utilized in the manufacture of potable alcohol from molasses. In these applications, the *T. reesei* TG enzyme will replace the current *A. niger*-derived TG. In practice, the *T. reesei* TG enzyme will not be present in the final food product.

In evaluating the Danisco *T. reesei* TG enzyme I considered the biology of *T. reesei*, information available in the peer-reviewed scientific literature, and the information that you provided regarding the safe lineage of the production organism and the cloning methodology that was utilized.

Trichoderma reesei is a non-pathogenic, non-toxigenic fungus that is widely used by enzyme manufacturers worldwide for the production of enzyme preparations for use in human food, animal feed, and numerous other industrial enzyme applications. The Danisco TG production strain, *T. reesei* strain TG #626, GICC 03289, was derived from a safe strain lineage that was developed by Danisco for the manufacture of a number of food and feed enzymes, including chymosin, xylanase, cellulase, glucoamylase, β-glucosidase/cellulase, alpha-amylase and acid

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fungal protease. Strains within this lineage have been subjected to rigorous safety evaluation in accordance with the Pariza-Johnson decision tree (Regulatory Toxicol. Pharmacol. 33: 173-186, 2001). Published literature, government laws and regulations, for example FR 64:28658-28362; 1999, reviews by expert panels such as FAO/WHO JECFA (1992), as well as Danisco's unpublished safety studies, support this conclusion.

The production organism, *T. reesei* strain TG #626, GICC 03289, was derived by modifying *T. reesei* strain RL-P37 using DNA from *Aspergillus niger* AGME9. *A. niger* AGME9 is the production organism for Genencor's current *A. niger* TG enzyme product, and like *T. reesei* strain RL-P37, *A. niger* AGME9 has undergone rigorous safety evaluation in accordance with the Pariza-Johnson decision tree. Accordingly, *A. niger* AGME9 is an appropriate organism from which to derive DNA for the production of food-grade enzymes. You have provided complete descriptions of the genetic modification methods used to clone the *A. niger* AGME9 TG gene into *T. reesei* strain RL-P37 to produce the new *T. reesei* strain TG #626, GICC 03289. You have also provided detailed information on the production methods, characterizations, and specifications for the TG enzyme product.

Based on this information, I conclude that the Danisco TG enzyme preparation produced by *T. reesei* strain TG #626, GICC 03289 using the manufacturing process that you describe is Generally Recognized as Safe (GRAS) safe for use under the conditions you have indicated in the conversion of malto-oligosaccharides in starch from grains to isomalto-oligosaccharides, and as one of the enzymes in an enzyme cocktail utilized in the production of potable alcohol from molasses. It is also my professional opinion that other qualified experts would 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
Professor of Food Science
Member, Michael W. Pariza Consulting LLC

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Appendix 6 – Composition of Tox Lot

CERTIFICATE OF ANALYSIS

Name of Test Article: **ACID FUNGAL PROTEASE**

Production/Strain Name: *Trichoderma reesei* AFP 55.3
Production Site: Jamsankoski, Finland

Designation of Lot Tested: AFP501K1
Description: Clear brown liquid

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

Results:

1. Activity: 2799 SAPU/g

2. Total and TCA Protein
   TCA Proteins: 78.6 mg/ml
   Total Proteins: 124.7 mg/ml
   % Total Organic Solids: 14.29 %
   (100% – moisture% – ash%)

3. Specific gravity: 1.042 g/ml

4. pH: 3.44

5. Inorganic materials
   % Ash: 0.17 %
   % moisture: 85.54 %

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

Analysis	Results
Total viable count	< 1CFU/ml
Coliform	< 1CFU/ml
E. Coli	negative/25 ml
Salmonella	negative/25 ml
Staphylococcus aureus	< 1 CFU/ml
Production strain	negative
Anaerobic sulfite reducers	negative
Antibiotic activity assay	negative

7. Mycotoxin analysis: Not applicable

8. Heavy metals analysis:
Conducted at Silliker Laboratories

Analysis	Results
Heavy metals as Pb	< 30 ppm
Arsenic	< 0.83 ppm
Lead	< 0.42 ppm
Mercury	< 0.05 ppm
Cadmium	< 0.17 ppm

9. Stability Data

Sample ID	Dilution	T = 0	T = 5 hours	% of T = 0
AFP501K1	straight	2732	2809	103
AFP501K1	1/2	2806	2897	103
AFP501K1	1/4	2723	2863	105

Refrigerator (4C) Stability

Sample ID	T = 0	T = 7 days	% of T = 0
AFP501K1	2732	2565	94

Freezer Stability

Sample ID	T = 0	T = 130 days	% of T = 0
AFP501K1	2732	2685	98

(b) (6) [redacted] 3/14/06
Christine Rechichi — Date
Corporate BioAnalytical Representative

(b) (6) [redacted] 14.3.2006
Anne Kantelinen — Date
Jokioinen, Finland

(b) (6) [redacted] March 14, 2006
Quang Q. Bui — Date
Study Sponsor Representative

BLANK PAGE(S) INSERTED FOR CORRECT PAGINATION

Farias, Bianca

From: Vincent Sewalt [vincent.sewalt@danisco.com]
Sent: Friday, December 18, 2009 4:10 PM
To: Martin, Robert L
Cc: Farias, Bianca; moraine.ramos@fda.hhs.gov; Alice Caddow
Subject: GRAS Notice on TG

Dear Dr. Martin,

On December 15, 2009 I submitted a supplement to our GRAS Notice for Transglucosidase Enzyme Preparation Derived from *Trichoderma reesei.* , which was originally submitted by Ms. Alice Caddow on December 4, 2009.

CFSAN received this supplementary submission just yesterday and Ms. Moraine Ramos and Ms. Bianca Farias called me this morning to verify whether a certificate of analysis enclosed as Appendix 6 marked "CONFIDENTIAL" is truly to be maintained confidential. I relayed to them that the data on the C of A is not confidential, but that I would need to double-check on the *T. reesei* strain ID number.

I have just verified this with our legal/IP department and indeed, **the Genencor International Culture Collection (GICC) Number: GICC 03243 is the only item on this Cof A that should be maintained confidential.**

I hope this satisfies the need.

I do wish to express my appreciation for the prompt and thoughtful handling of this submission by your staff.

Sincerely,

Vincent Sewalt, PhD
Director of Regulatory Affairs
Genencor, a Danisco Division
925 Page Mill Road
Palo Alto, CA 94304
Tel 650-846-5861 | Cel 650-799-0871

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Farias, Bianca

From: Martin, Robert L
Sent: Tuesday, January 05, 2010 2:09 PM
To: 'Vincent Sewalt'
Cc: Farias, Bianca; Alice Caddow; Ramos-Valle, Moraima
Subject: RE: GRAS Notice on TG

Dr. Sewalt, we have reviewed your claim of confidentiality for the GICC number. We do not agree that this number is confidential as it is a source identifier for anyone who may need to reference this material. This determination is consistent with the way things are handled with the American Type Culture Collection.

Please feel free to call me if you have any questions.

Thanks.
Robert L. Martin
301-436-1219

From: Vincent Sewalt [mailto:vincent.sewalt@danisco.com]
Sent: Friday, December 18, 2009 4:10 PM
To: Martin, Robert L
Cc: Farias, Bianca; moraine.ramos@fda.hhs.gov; Alice Caddow
Subject: GRAS Notice on TG

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Sincerely,

Vincent Sewalt, PhD
Director of Regulatory Affairs
Genencor, a Danisco Division
925 Page Mill Road
Palo Alto, CA 94304

000080

Tel 650-846-5861 | Cel 650-799-0871

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Farias, Bianca

From: Vincent Sewalt [vincent.sewalt@danisco.com]
Sent: Tuesday, January 05, 2010 2:48 PM
To: Martin, Robert L
Cc: Alice Caddow; Farias, Bianca; Ramos-Valle, Moraima
Subject: GRAS Notice on TG
Attachments: CofA AFP.pdf

Dear Dr. Martin,

As discussed via phone this morning, please find attached an amended C of A for our *T. reesei* Transglucosidase GRAS Notice. Please replace the earlier version on page 49 of the submission with the attached version. Thank you.

Best regards,

Vincent Sewalt, PhD
Director of Regulatory Affairs
Genencor, a Danisco Division
925 Page Mill Road
Palo Alto, CA 94304
Tel 650-846-5861 | Cel 650-799-0871

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(b) (6)

3/14/06

Christine Rechichi
Corporate BioAnalytical Representative

Date

(b) (6)

14.3.2006

Anne Kantelinen
Iamsankoski, Finland

Date

(b) (6)

March 14, 2006

Quang Q. Bui
Study Sponsor Representative

Date

Farias, Bianca

From: Martin, Robert L
Sent: Wednesday, January 06, 2010 7:06 AM
To: 'Vincent Sewalt'
Cc: Alice Caddow; Farias, Bianca; Ramos-Valle, Moraima
Subject: RE: GRAS Notice on TG

Dr. Sewalt, page 2 of the C of A is marked "Confidential." Based on previous statements, we do not believe that you intended that to be the case. If so, please remove the confidential stamp and resubmit page 2.

Thanks.
Robert L. Martin
301-436-1219

From: Vincent Sewalt [mailto:vincent.sewalt@danisco.com]
Sent: Tuesday, January 05, 2010 2:48 PM
To: Martin, Robert L
Cc: Alice Caddow; Farias, Bianca; Ramos-Valle, Moraima
Subject: GRAS Notice on TG

Dear Dr. Martin,

As discussed via phone this morning, please find attached an amended C of A for our *T. reesei* Transglucosidase GRAS Notice. Please replace the earlier version on page 49 of the submission with the attached version. Thank you.

Best regards,

Vincent Sewalt, PhD
Director of Regulatory Affairs
Genencor, a Danisco Division
925 Page Mill Road
Palo Alto, CA 94304
Tel 650-846-5861 | Cel 650-799-0871

This is an e-mail from Danisco and may contain confidential information. If you are not the intended recipient and you receive this e-mail by mistake, you are not allowed to use the information, to copy it or distribute it further.
Please notify us and return it to Danisco by e-mail and delete all attachments. Thank you for your assistance.

Farias, Bianca

From: Martin, Robert L
Sent: Wednesday, January 06, 2010 8:31 AM
To: Farias, Bianca
Subject: FW: GRAS Notice on TG
Attachments: CofA AFP.pdf

Thanks.
Robert L. Martin
301-436-1219

From: Vincent Sewalt [mailto:vincent.sewalt@danisco.com]
Sent: Wednesday, January 06, 2010 8:23 AM
To: Martin, Robert L
Cc: Alice Caddow; Georgette Shintaku
Subject: RE: GRAS Notice on TG

Dr. Martin - my apologies for the oversight, a corrected version of the complete C of A is attached here:

Best regards,

Vincent Sewalt,

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Please notify us and return it to Danisco by e-mail and delete all attachments. Thank you for your assistance.

"Martin, Robert L" <Robert.Martin@fda.hhs.gov>

Posted date : 01/06/2010 07:05:47 AM EST

To "Vincent Sewalt" <vincent.sewalt@danisco.com>
cc "Alice Caddow" <alice.caddow@danisco.com>, "Farias, Bianca" <Bianca.Farias@fda.hhs.gov>, "Ramos-Valle, Moraima" <Moraima.Ramos-Valle@fda.hhs.gov>
Subject RE: GRAS Notice on TG

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Genencor International, Inc.

CERTIFICATE OF ANALYSIS

Name of Test Article: **ACID FUNGAL PROTEASE**

Production/Strain Name: *Trichoderma reesei* AFP 55.3
Production Site: Jamsankoski, Finland
Genencor International Culture Collection Number: **GICC 03243**

Designation of Lot Tested: AFP501K1
Description: Clear brown liquid

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

Results:

1. Activity: 2799 SAPU/g

2. Total and TCA Protein
   TCA Proteins: 78.6 mg/ml
   Total Proteins: 124.7 mg/ml
   % Total Organic Solids: 14.29 %
   (100% – moisture% – ash%)

3. Specific gravity: 1.042 g/ml

4. pH: 3.44

5. Inorganic materials
   % Ash: 0.17 %
   % moisture: 85.54 %

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

Analysis	Results
Total viable count	< 1CFU/ml
Coliform	< 1CFU/ml
E. Coli	negative/25 ml
Salmonella	negative/25 ml
Staphylococcus aureus	< 1 CFU/ml
Production strain	negative
Anaerobic sulfite reducers	negative
Antibiotic activity assay	negative

7. Mycotoxin analysis: Not applicable

8. Heavy metals analysis:
Conducted at Silliker Laboratories

Analysis	Results
Heavy metals as Pb	< 30 ppm
Arsenic	< 0.83 ppm
Lead	< 0.42 ppm
Mercury	< 0.05 ppm
Cadmium	< 0.17 ppm

9. Stability Data

Sample ID	Dilution	T = 0	T = 5 hours	% of T = 0
AFP501K1	straight	2732	2809	103
AFP501K1	1/2	2806	2897	103
AFP501K1	1/4	2723	2863	105

Refrigerator (4C) Stability

Sample ID	T = 0	T = 7 days	% of T = 0
AFP501K1	2732	2565	94

Freezer Stability

Sample ID	T = 0	T = 130 days	% of T = 0
AFP501K1	2732	2685	98

(b) (6) [redacted] 3/14/06

Christine Rechichi
Corporate BioAnalytical Representative
Date

(b) (6) [redacted] 14.3.2006

Anne Kantelinen
Jamsankoski, Finland
Date

(b) (6) [redacted] March 14, 2006

Quang Q. Bui
Study Sponsor Representative
Date

SUBMISSION END